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The Fragile X Mental Retardation Protein Developmentally Regulates the Strength and Fidelity of Calcium Signaling in *Drosophila* Mushroom Body Neurons

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Abstract

Fragile X syndrome (FXS) is a broad-spectrum neurological disorder characterized by hypersensitivity to sensory stimuli, hyperactivity and severe cognitive impairment. FXS is caused by loss of the *fragile X mental retardation 1* (FMR1) gene, whose FMRP product regulates mRNA translation downstream of synaptic activity to modulate changes in synaptic architecture, function and plasticity. Null *Drosophila* FMR1 (*dfmr1*) mutants exhibit reduced learning and loss of protein synthesis-dependent memory consolidation, which is dependent on the brain Mushroom Body (MB) learning and memory center. We targeted a transgenic GFP-based calcium reporter to the MB in order to analyze calcium dynamics downstream of neuronal activation. In the *dfmr1* null MB, there was significant augmentation of the calcium transients induced by membrane depolarization, as well as elevated release of calcium from intracellular organelle stores. The severity of these calcium-signaling defects increased with developmental age, although early stages were characterized by highly variable, low fidelity calcium regulation. At the single neuron level, both calcium transient and calcium store release defects were exhibited by *dfmr1* null MB neurons in primary culture. Null *dfmr1* mutants exhibit reduced brain mRNA expression of calcium-binding proteins, including calcium buffers calmodulin and calbindin, predicting that the inability to appropriately sequester cytosolic calcium may be the common mechanistic defect causing calcium accumulation following both influx and store release. Changes in the magnitude and fidelity of calcium signals in the absence of dFMRP likely contribute to defects in neuronal structure/function, leading to the hallmark learning and memory dysfunction of FXS.

Keywords

Fragile X syndrome; cognitive impairment; autism; learning; memory; synapse; translation; mRNA; calcium buffering proteins

Introduction

Fragile X syndrome (FXS) is the most common heritable mental retardation and autism disorder (Belmonte and Bourgeron, 2006; Clifford et al., 2007; Cohen et al., 2005; Hagerman

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et al., 2005; Rogers et al., 2001), manifesting multiple activity-regulation defects including hyperactivity and childhood epileptic seizures (Freund and Reiss, 1991; Sabaratnam et al., 2001; Torrioli et al., 2008). Consistently, FXS animal models display hyperactivity, audiogenic seizures and over-elaborated synaptic processes that are normally regulated in activity-dependent mechanisms (Comery et al., 1997; Galvez and Greenough, 2005; Galvez et al., 2005; Grossman et al., 2006; Nimchinsky et al., 2001). In addition, FXS models show defects in activity-dependent synaptic function, including brain region specific defects in long-term potentiation (LTP) and depression (LTD) (Godfraind et al., 1996; Huber et al., 2002; Koekkoek et al., 2005; Zhao et al., 2005). Both activity-regulated synaptic structure and function defects are dependant on the stage of neural circuit maturation (Bureau et al., 2008; Harlow et al., 2010; Larson et al., 2005).

FXS results from the loss of Fragile X Mental Retardation Protein (FMRP) whose function is completely conserved from *Drosophila* to humans (Coffee et al., 2010). In both mammals and *Drosophila*, FMRP functions downstream of synaptic activity, including metabotropic glutamate receptor (mGluR) signaling and likely other neurotransmitter-gated Gq signaling mechanisms (Bear et al., 2004; Pan et al., 2008; Repicky and Broadie, 2009; Volk et al., 2007). Likewise, FMRP expression is positively regulated by activity and repressed under conditions of reduced activity (Gabel et al., 2004; Tessier and Broadie, 2008; Valentine et al., 2000). FMRP associates with polyribosomes in an activity-dependent manner to regulate protein synthesis (Aschrafi et al., 2005; Khandjian et al., 2004; Nosyreva and Huber, 2006; Stefani et al., 2004). In all species, FMRP functions in mRNA translocation/stability and translational repression, most evidently during activity stimulation (Antar et al., 2004; Park et al., 2008; Todd et al., 2003; Zalfa et al., 2007; Zhang et al., 2001). Membrane depolarization leads to increases in protein synthesis and mobilization of FMRP granules into synaptic regions (Antar et al., 2004; Muddashetty et al., 2007). Thus, FMRP both responds to neuronal activity levels and mediates protein changes that modulate the activity-dependent mechanisms directing morphological and functional changes in neural circuits.

In the *Drosophila* brain Mushroom Body (MB) learning and memory center, dFMRP is required for activity-dependent synapse number regulation and protein synthesis-dependent long-term memory formation (Bolduc et al., 2008; Pan et al., 2008; Pan et al., 2004; Tessier and Broadie, 2008). In *dfmr1* null animals, the activity-regulated pruning of MB synapses fails during the immediate post-eclosion period of use-dependent process refinement (Tessier and Broadie, 2008). In contrast, synapse elimination at maturity is over-exuberant in *dfmr1* null MB neurons, resulting in a significant reduction in adult process elaboration. The expression of dFMRP is dependent on developmental timing and neuronal activity regulates both dFMRP expression and synapse elimination (Tessier and Broadie, 2008). Therefore, we hypothesized that dFMRP has developmental stage-specific functions which regulate the efficacy of neuronal activity in developing and mature circuits. The most obvious and important readout of neuronal activity is the regulation of cytosolic calcium dynamics, which are required for FMRP-dependent processes ranging from synaptogenesis and synaptic refinement during development, to the structural and functional synaptic plasticity manifested at maturity (Lnenicka et al., 2006; Lohmann, 2009; Peng and Guo, 2007; Reiff et al., 2005).

In this study, we employ a transgenic GFP-based calcium reporter targeted to the *Drosophila* Mushroom Body to monitor calcium dynamics both during development and at maturity. We show that *dfmr1* null mutants exhibit development stage-specific defects in activity-dependent calcium influx and calcium store release dynamics. These defects are significant only in mature *dfmr1* MB neurons, but the developing neurons exhibit a striking loss of calcium signaling fidelity revealed by a significant increase in response variability during MB circuit maturation. Null *dfmr1* mutant brains exhibit altered mRNA expression of key calcium-binding proteins in a development stage-specific profile, which may explain the

enhanced cytosolic calcium signals during both transient and store release mechanisms. These data suggest that defective activity-dependent calcium regulation caused by the absence of dFMRP may contribute to structural and functional defects underlying FXS learning/memory impairments.

Materials and Methods

Drosophila Stocks

The mutant and transgenic stocks used in these experiments were generated by standard genetic recombination techniques. The following genotypes were used for molecular studies: control (*w¹¹¹⁸*) and *dfmr1* null (*w¹¹¹⁸; frt82b, dfmr1^{50M}*). For all calcium imaging studies, the following genotypes were used: control (UAS-gCAMP1.3/+; OK107-GAL4/+), two independent *dfmr1* nulls (UAS-gCAMP1.3/+; *frt82b, dfmr1^{50M}*; OK107-GAL4/+ and UAS-gCAMP1.3/+; *dfmr1³*; OK107-GAL4/+) and exogenous wildtype *dfmr1* expression in the null background (UAS-gCAMP1.3/+; *dfmr1^{50M}*, UAS-dFMR1(9557-3)/*dfmr1^{50M}*; OK107-GAL4/+). The UAS-gCAMP1.3 transgenic line was kindly provided by Mike Adams (University of California-Riverside) (Nakai et al., 2001).

Immunocytochemistry

Immunological analyses were performed as described previously (Tessier and Broadie, 2008). Briefly, brains were dissected in 1XPBS, fixed in 4% paraformaldehyde for 30 minutes, then blocked with 1XPBS, 1% BSA and 0.2% triton-X 100. Preparations were incubated in primary antibody (rabbit anti-GFP; Abcam Ab 290; 1:50,000, Sigma dFMRP clone 6A15; 1:200) at 4°C for 12–16 hours before washing with blocking buffer. Preparations were then incubated in secondary antibody (Alexa 568 anti-rabbit IgG; Alexa 488 anti-rabbit IgG; Alexa 568 anti-mouse IgG; Molecular Probes; 1:1000,) at 25°C for 2 hours. Brains were mounted in Fluoromount G, and imaged on a Zeiss Meta 510 confocal microscope using appropriate filters.

Calcium imaging

For basal calcium measurements, brains were dissected in physiological saline (70mM NaCl, 5mM KCl, 5mM MgCl₂, 1.5mM CaCl₂, 10mM NaHCO₃, 115mM sucrose, 5mM D-trehalose, 5mM Hepes, pH 7.2). Acutely dissected brains were imaged immediately using a 20X air objective on a Zeiss Meta 510 confocal microscope using a 505–530 bandpass filter. A complete Z-series was taken through the Mushroom Body to include the entire axonal lobes. Identical settings were used to image all genotypes. Basal calcium levels were measured as the average fluorescence of the gCAMP reporter under no stimulation conditions. To measure calcium transients, brains were dissected in a dish containing 4mL of the above saline. Brains were then imaged immediately under a 40X water immersion objective with the pinhole set to maximum. Only regions of interest around the MB axonal lobes were imaged using 256 × 256 pixel settings to obtain scan speeds of <300ms per scan. Continuous scanning was done for 1 minute to establish a baseline fluorescence and then 3M KCl was added to the dish to a final concentration of 70mM to depolarize the preparations (Yu et al., 2003). Scanning continued for 1 minute after depolarization. For release of calcium from internal stores, brains were dissected in zero calcium containing saline (70mM NaCl, 5mM KCl, 5mM MgCl₂, 10mM NaHCO₃, 115mM sucrose, 5mM D-trehalose, 5mM Hepes, pH 7.2) and placed on a glass slide. Complete Z-series through the axonal lobes were imaged immediately using a 20X air objective. Imaging was done in 1 minute intervals for 3 minutes to set the basal fluorescence intensity. Immediately after the third scan, the brain was depolarized with saline containing 70mM KCl. Image acquisition was then continued at 1 minute intervals for 15 minutes.

Brain primary neuronal culture

Primary neuron cultures were generated from dissociated 72 hour old pupal brains as previously described (Phillips et al., 2008; Sicaeros et al., 2007; Vijayakrishnan et al., 2009). Briefly, brains were dissected in dissecting saline (DS) (6.9mM NaCl, 0.3mM KCl, 9μM Na₂HPO₄, 11μM KH₂PO₄, 33.3mM glucose, 43.8mM sucrose 0.28mM Hepes, pH 7.4) and then incubated for 15 minutes at 25°C in an enzyme solution containing 50U papain and L-Cysteine in DS. Brains were washed 3X with DS and once with DMEM containing 20mM Hepes and 200mM L-glutamine. Each brain was then dissociated using a 30 gauge needle and triturated through a pulled glass micropipette on a concanavalin A/laminin coated cover slip. Cover slips were placed in 35mm plastic dishes and allowed to settle for 30 minutes at 25°C. Plates were then flooded with 1.5mL of DMEM supplemented with transferrin (100μg/mL), putrescine (100μM), selenium (30nM), progesterone (20ng/mL), insulin (50μg/mL), 20-hydroxyecdysone (2.1μM). Cultures were maintained at 20–25°C and 5% CO₂ for 14–16 hours and then fed 0.5mL glial conditioned media. On the fifth day of culturing, the medium was replaced with a 3:1 ratio of supplemented DMEM to glial conditioned media. Experiments were performed on either day 3 or day 6 *in vitro*. Calcium imaging was done as for whole brains (above). A 63X water immersion objective was used to image only a single field for each brain. Only OK107 GFP-positive neurons were quantified, however with no other selection criteria used including the structure of individual cells, cell density or cell-to-cell contacts within the heterogeneous cultures.

Calcium quantifications

LSM region of interest software tools were used to measure average fluorescence values. All quantifications were normalized to initial fluorescence baseline which was taken as the average value 40–50 seconds into the scan for transients. For depolarization transient measurements; (1) the amplitude was the magnitude between the lowest and highest values after stimulation, and (2) the rise to peak was the time period between the lowest and highest values after stimulation. To quantify store release, summed intensity stacks were made from each LSM acquired Z-series at each time point using Image J software. The scan done immediately before depolarization was used as the baseline and regions of interest were drawn around each MB lobe (intact brain) or isolated MB neurons (cell culture). Each region was quantified independently and then averaged for each brain or culture. MBs or cells were considered to respond only if they showed a fluorescence increase of at least 5% over baseline and maintained their maximal value $\pm$ 5% through the final time point. Any sample outside 3 standard deviations was considered an outlier and was not used in analyses. All significance values were obtained using a two-sided student's t-test for each time point. Significance of variance was calculated using two-sided F-tests.

Quantitative RT-PCR

RNA was isolated from *Drosophila* heads at the indicated time points, DNase treated, and reverse transcribed as previously described (Tessier and Broadie, 2008). Quantitative PCR was carried out using SYBR Green Jumpstart Taq Ready mix (Sigma) on 1μL of cDNA and 0.5μM of each of the following primer pairs:

gapdh2:	5'-CCGATGCGACCAAATCCATTGATA-3'
	5'-CGCTCAAAATTTCTCAGCCATCAC-3'
frequenin1:	5'-CCCAACGGCCTGCTACGAGACAA-3'
	5'-GCGTTATGTAACCATCGTTGTCCA-3'
frequenin2:	5'-GGCTTTCTCAAAGACTGTCCGAATG-3'
	5'-GCGTCCACTATGTTGTACATCTCCT-3'

calmodulin: 5'-CGCAGCGGATTAAACTTCGTCACCTCTAGCAACA-3'
 5'-GGCATCAACCTCGTTGATCATGTCCTG-3'
 calbindin: 5'-GCCAACAAGGACGGACGTCTGCAGTTGTC-3'
 5'-GGCATCATAGTCGTCCTTCTTGACCAACT-3'.

Cycling parameters were 95°C (3 mins), 95°C (30 secs), 60°C (30 secs), and 72°C (30 secs) for 40 cycles each in a BioRad IQ5 Thermal Cycler. Each experiment consisted of 4–6 biological replicates plated in duplicate. Results are the average of 3 separate experiments. Quantification was performed using delta delta Ct comparison with *gapdh2* as the internal standard (Dussault and Pouliot, 2006). Results are expressed as fold changes in mutants compared to controls at each time point.

Results

Ca²⁺ levels differentially increase during *dfmr1* null Mushroom Body maturation

The *Drosophila* Mushroom Body (MB) learning and memory center manifests cell autonomous, activity-dependent functional and structural defects in the absence of dFMRP (Pan et al., 2008; Pan et al., 2004; Tessier and Broadie, 2008). These defects are presumed responsible for the loss of learning and memory consolidation in the *Drosophila* FXS model, a defect shared with the mouse model and FXS patients (Bennetto et al., 2001; Bolduc et al., 2008; Choi et al., 2010; Zhao et al., 2005). Activity-dependent calcium signaling is absolutely required for these processes, and therefore the maintenance of calcium homeostasis is tightly controlled in neurons (Lohmann, 2009). We hypothesized that MB calcium signaling may be impaired in *dfmr1* mutants to cause both cellular and behavioral defects. To examine this possibility, relative calcium levels were first assayed in intact brain MBs from control and *dfmr1* nulls. Previously reported *dfmr1* phenotypes segregate into two classes; 1) transient during development and 2) persistent at maturity. Therefore, calcium analyses were divided into the two relevant time points; 1) 0–3 hours post-eclosion, a period of activity-dependent MB circuit refinement which coincides with maximal dFMRP expression, and 2) 4 days post-eclosion, a mature time point of reduced dFMRP expression (Tessier and Broadie, 2008). The transgenic GFP-based fluorescent calcium reporter gCAMP was used to assay relative calcium levels (Nakai et al., 2001). Calcium binding to a calmodulin domain results in a conformational change to increase reporter GFP fluorescence (Akerboom et al., 2009). The MB-specific OK107-GAL4 was used to target UAS-gCAMP specifically to the MB circuit (Fig. 1).

Control and *dfmr1* null brains were acutely dissected in 1.5mM Ca²⁺ saline and imaged immediately on a confocal microscope. All processing and microscope settings were kept identical for each genotype and age group (Fig. 1A). The gCAMP Ca²⁺ reporter fluorescence showed no significant differences between control and *dfmr1* null MBs, in both 0–3 hour old animals (control: 235±23, n=13; *dfmr1*^{50M}: 271±34, n=13; p=0.38) and 4 day old animals (control: 515±40, n=13; *dfmr1*^{50M}: 440±50, n=11; p=0.25). However, a significant (p<0.001) increase in Ca²⁺ reporter fluorescence was observed in both genotypes at the mature 4 day time point compared to the immature 0–3 hour time point (Fig. 1B). Control MBs showed a 120% increase in calcium level fluorescence over this developmental time course, whereas *dfmr1* null MBs showed only a 62% increase. To determine if this fluorescence change was simply a result of increased gCAMP expression, brains from each genotype and each time point were labeled with anti-GFP antibodies to determine relative reporter expression (Fig. 1C). There were no significant differences in gCAMP reporter expression between genotypes at 0–3 hours (control: 263±44, n=12; *dfmr1*^{50M}: 320±25, n=12; p=0.27) or 4 days (control: 335±43, n=17; *dfmr1*^{50M}: 267±32, n=12; p=0.25; Fig. 1D). Therefore, basal Ca²⁺ concentrations in the MB circuit increase as a function of development time, differentially in *dfmr1* nulls compared to

controls, although the basal Ca^{2+} concentration in MB neurons at any given time point is not detectably altered in *dfmr1* null animals.

dFMRP regulates activity-dependent Ca^{2+} transients in the Mushroom Body

In both *Drosophila* and mammals, FMRP functions in activity-dependent mechanisms, and membrane depolarization has been used repeatedly to identify FMRP-dependent defects (Antar et al., 2004; Bear et al., 2004; Muddashetty et al., 2007; Nosyreva and Huber, 2006; Repicky and Broadie, 2009; Tessier and Broadie, 2008). Since external calcium influx is a key signaling event, we first examined depolarization-dependent calcium transients in control and *dfmr1* null MBs from intact brains (Fiala and Spall, 2003; He et al., 2009; Kuromi et al., 2004; Reiff et al., 2005; Wang et al., 2004b; Yu et al., 2006; Yu et al., 2003). To confirm specificity to the *dfmr1* locus, we utilized two independently generated alleles of *dfmr1* (*dfmr1*^{50M} and *dfmr1*³) for all experiments.

In 1.5mM Ca^{2+} saline, brains were first imaged continuously for 1 minute to establish a baseline (pre-stimulation), and then acutely depolarized with 70mM KCl (Fig. 2A). The result of this protocol is a brief decrease in reporter intensity due to osmotic shock, then a sharp fluorescence increase as Ca^{2+} enters the neurons (transient high), followed by a rapid Ca^{2+} decline (post-transient) (Fiala and Spall, 2003). Two parameters of the Ca^{2+} fluorescence curve were quantified for comparison between control and *dfmr1* null MBs (Fig. 2B); 1) the amplitude of the Ca^{2+} transient peak and 2) the rise to Ca^{2+} peak time. The decline phase of the curve was not measured, as it was highly variable in both controls and mutants using this experimental procedure. To test for development stage-specific roles of dFMRP, two time points were compared; 1) the immature MB immediately post-eclosion (0–3 hours), and 2) the mature, adult MB (4 days). Figure 2 shows quantified Ca^{2+} fluorescence transient amplitudes (Fig. 2C) and time to peak Ca^{2+} values (Fig. 2D) for all genotypes at both time points.

The mean Ca^{2+} transient amplitude was not significantly different between control and *dfmr1* nulls at 0–3 hours post-eclosion (control: $75.1\% \pm 3.0$, $n=19$; *dfmr1*^{50M}: $77.1\% \pm 7.1$, $n=20$; $p=0.7$; Fig. 2C). However, wild-type MBs exhibited reduced Ca^{2+} transient amplitudes as a function of developmental age, whereas *dfmr1* nulls did not. Therefore, Ca^{2+} transient amplitudes in *dfmr1* mutant MBs were significantly (10–15%) larger than controls at 4 days of age (control: $66.4\% \pm 2.7$, $n=22$; *dfmr1*^{50M}: $75.5\% \pm 2.7$, $n=21$; $p=0.02$; *dfmr1*³: $73.9\% \pm 2.2$, $n=24$; $p=0.04$; Fig. 2C). Similarly, the length of time it takes for the Ca^{2+} signal to rise to its peak amplitude was not quite significantly different ($p=0.06$) in *dfmr1* neurons compared to controls in the immature (0–3 hr) MB (control: 5.2 ± 0.8 , $n=19$; *dfmr1*^{50M}: 8.4 ± 1.5 , $n=20$; Fig. 2D). However, at 4 days, there was a significant increase (33%) in the length of time it takes for *dfmr1*^{50M} Ca^{2+} signals to rise to peak compared to controls (control: 4.5 ± 0.2 , $n=22$; *dfmr1*^{50M}: 6.0 ± 0.6 , $n=21$; $p=0.02$; Fig. 2D). Note that the rise time is faster for both genotypes at the mature time point. The rise time defect, however, was not observed in the *dfmr1*³ allele, and is specific to the genetic background of the *dfmr1*^{50M} allele (*dfmr1*³: 4.3 ± 0.4 , $n=24$; $p=0.7$; Fig. 2D). Nonetheless, the control of depolarization-dependent Ca^{2+} influx dynamics is compromised in *dfmr1* mutant neurons, and the defect becomes progressively more severe with the maturation of the MB circuit.

To further investigate the role dFMRP may play in regulating calcium transients, we measured transient amplitudes following MB-targeted dFMRP expression in the *dfmr1*^{50M} null background (OK107-GAL4/+; UAS-dFMR1/+; *dfmr1*^{50M}/*dfmr1*^{50M}; hereafter referred to as dFMR1). In this combination, the entire animal lacks dFMRP except the brain MB (Supplemental Fig. 1). By four days post-eclosion, this produced a significant overexpression condition, likely due to the normal developmental downregulation of endogenous dFMRP which is not mimicked by the OK107 driver (Tessier and Broadie, 2008). In this overexpression condition, calcium transient amplitudes were significantly augmented (control: $66.4\% \pm 2.7$,

n=22; dFMR1: $95.1\% \pm 4.5$, n=19; $p < 0.001$) consistent with neuronal overexpression phenotypes reported previously (Zhang et al., 2001). Moreover, the delayed rise to peak time observed in *dfmr1*^{50M} animals was restored to normal, with no significant difference to controls (control: 4.5 ± 0.2 , n=22; dFMR1: 5.0 ± 0.6 , n=19; $p = 0.4$). Thus, increasing dFMRP expression significantly alters calcium influx transient dynamics.

dFMRP regulates activity-dependent Ca^{2+} transients in isolated MB Kenyon cells

Null *dfmr1* Mushroom Bodies exhibit gross structural abnormalities ranging from aberrant midline crossing and loss of lobe structures, to single cell branching overgrowth and loss of activity-dependent synapse pruning (McBride et al., 2005; Michel et al., 2004; Pan et al., 2004; Tessier and Broadie, 2008). It was therefore possible that these structural defects might contribute to the changes in Ca^{2+} transients and therefore compromise interpretation of results. To eliminate this variability from our analysis, and to examine Ca^{2+} responses at the single neuron level, we performed the same series of experiments on primary MB neuronal cultures (Jiang et al., 2005; Oh et al., 2008; Sicaeros et al., 2007; Su and O'Dowd, 2003). Cultures were made from animals expressing the gCAMP reporter only in MB Kenyon cells (OK107-GAL4 X UAS-gCAMP). Neurons were grown for either 1) 3 days *in vitro* (DIV) (a time prior to detectable synaptic development) or 2) 6 DIV (a mature time point with abundant synaptic connections) (Oh et al., 2008). Neurons were acutely depolarized with 70mM KCl, using the same protocol described above (Fig. 3).

Compared to measurements in the intact brain MB, rise to Ca^{2+} peak times were extremely rapid for both control and mutant genotypes (Fig. 3A,B). With our experimental setup, it was not possible to measure these rise times with sufficient accuracy to report on *dfmr1* phenotypes, and therefore only the amplitude of the Ca^{2+} transients was measured. Similar to results in the immature intact brains, transient amplitudes from *dfmr1* null MB neurons were not significantly different than controls at 3 DIV (controls: $128.8\% \pm 7.7$, n=68 cells from 10 brains; *dfmr1*^{50M}: $129.0\% \pm 9.6$, n=67 cells from 10 brains; $p = 0.99$) (Fig. 3C). In contrast, consistent with the developmental profile seen in intact brains, *dfmr1* null MB neurons from mature 6 DIV cultures exhibited a highly significant increase in Ca^{2+} transient amplitudes compared to controls (Fig. 3C). Mutant neurons from two independent *dfmr1* alleles showed a >20% increase in depolarization-dependent Ca^{2+} influx (control: $124.1\% \pm 4.5$, n=49 cells from 10 brains; *dfmr1*^{50M}: $150.3\% \pm 6.7$, n=44 cells from 7 brains; $p = 0.004$; *dfmr1*³: $166.8\% \pm 7.2$, n=72 cells from 11 brains; $p < 0.0001$). Therefore, the Ca^{2+} influx caused by membrane depolarization is increased by the loss of dFMRP, not as the result of changes in MB neuronal architecture or defects at the circuit level, but rather because of defects in the processing of neuronal activity within each MB neuron. In the dFMR1 overexpression condition (Supplemental Fig. 1), Ca^{2+} transient amplitudes were ~20% larger than control neurons at 6 DIV (control: $124.1\% \pm 4.5$, n=49 cells from 10 brains; dFMR1: $153.5\% \pm 7.3$, n=90 cells from 11 brains; $p = 0.003$). These data suggest that the extent to which dFMRP regulates Ca^{2+} transients in MB neurons is dependent on developmental timing and the context of MB circuit connectivity.

dFMRP regulates activity-dependent Ca^{2+} release from MB internal stores

A second source of calcium signals is regulated Ca^{2+} release from intracellular storage sites (Meldolesi and Pozzan, 1998; Verkhratsky, 2002). During neuronal processing, cytosolic calcium concentrations are regulated by ionic mobilization into and out of storage organelles, including the endoplasmic reticulum (Babai et al., 2009; Conti et al., 2004). Therefore, we wanted to examine whether this second component of calcium homeostatic control was also affected by loss of dFMRP. For these experiments, brains were dissected in Ca^{2+} -free saline, a condition in which long-lasting Ca^{2+} release from stores occurs in the absence of extracellular Ca^{2+} influx (Kuromi and Kidokoro, 2002; Ryglewski et al., 2007; Sanyal et al., 2005). Brains

were first imaged to obtain baseline MB fluorescence and then depolarized with 70mM KCl saline, with MB imaging at 1 minute intervals for 15 minutes (Fig. 4). As above, experiments were performed at two time points; immediately after eclosion, and at adult maturity.

Following membrane depolarization, there was a general trend throughout the imaging time course of increasing gCAMP reporter fluorescence intensity, indicating long-lasting release of Ca^{2+} from organelle sequestration (Fig. 4A). This increase in cytosolic Ca^{2+} levels was observed in both control and *dfmr1* null MBs. At the MB circuit refinement time point of 0–3 hours post-eclosion, there was a clear trend towards elevated depolarization-dependent Ca^{2+} release in the absence of dFMRP, although the difference between control and *dfmr1* null was not statistically significant (Fig. 4B; control $n=20$, *dfmr1*^{50M} $n=25$). However, at the mature time point of 4 days, accelerated Ca^{2+} elevation became significant in both *dfmr1* null alleles compared to controls (Figs. 4C and D). For example, after 10 minutes post-stimulation, *dfmr1*^{50M} null MBs showed on average >60% increased Ca^{2+} fluorescence at each imaging point compared to controls (p values from 0.02; control ($n=20$), *dfmr1*^{50M} ($n=19$)). Using the *dfmr1*³ allele, this effect was more pronounced as significant increases in Ca^{2+} were evident by 4 minutes post-stimulation (p values from 0.03–0.007; control ($n=20$), *dfmr1*³ ($n=21$)). Thus, loss of dFMRP results in an increase in the cytosolic accumulation of Ca^{2+} released from internal stores over time.

We next assayed MB-targeted dFMRP expression in the *dfmr1*^{50M} null background (dFMR1; Supplemental Fig. 1). Opposing the phenotype observed in the null, Ca^{2+} reporter fluorescence intensity post-stimulation was significantly decreased in dFMR1 overexpressing animals relative to controls (Fig. 4E). Between 30–40% reductions in calcium fluorescence were detected during the last 4 minutes of the 15 minute experimental time course (p from 0.03–0.007, control ($n=20$), dFMR1 ($n=20$); Fig. 4E). Therefore, loss of dFMRP increases the release of calcium from intracellular stores, while excessive dFMRP causes the opposite effect. These Ca^{2+} homeostasis defects were revealed at the level of the MB axonal lobes in the context of all cells and the complete, intact circuit.

dFMRP regulates Ca^{2+} store release dynamics in isolated MB Kenyon cells

As with the analysis of Ca^{2+} transients, a potential complicating factor of measuring store release is *dfmr1* null MB overgrowth (Michel et al., 2004; Pan et al., 2004; Tessier and Broadie, 2008). To test whether differences in neuronal architecture influenced interpretation of Ca^{2+} dynamics in the whole brain, we performed imaging experiments on primary neuronal cultures derived from *dfmr1* null and control brains as described above (Jiang et al., 2005; Sicaeros et al., 2007; Su and O'Dowd, 2003). Cultures were grown for either 3 or 6 days *in vitro*, then placed in physiological saline lacking calcium, limiting the calcium source to intracellular stores within the neurons. The isolated MB neurons were first imaged to determine baseline fluorescence and then acutely depolarized in 70mM KCl saline, with imaging at 1 minute intervals for a total time course of 15 minutes (Fig. 5).

The Ca^{2+} reporter fluorescence was readily imaged in individual MB Kenyon cells, with membrane depolarization-dependent accumulation of cytosolic Ca^{2+} occurring over the course of the 15 minute imaging trial (Fig. 5A). For immature cultures at 3 DIV, *dfmr1*^{50M} cultures showed significant elevation of fluorescence by 10 minutes post-stimulation (p from 0.03–0.008, control ($n=60$ cells from 10 brains); *dfmr1*^{50M} ($n=89$ cells from 12 brains); Fig. 5B). The maximal magnitudes of fluorescence increases from immature cultures, however, were significantly reduced for both controls (~56%) and mutants (~62%) relative to more mature 6 DIV cultures (compare Figs. 5B and C). This finding suggests that the processing of calcium from internal stores is significantly different depending on developmental age. 6 DIV cultures showed a highly significant ($p=0.004$) efflux of cytosolic Ca^{2+} at the earliest time-point (1 minute post-stimulation), in both *dfmr1*^{50M} and *dfmr1*³ alleles, that was not observed in

controls (control (n=39 cells from 10 brains); *dfmr1^{50M}* (n=50 cells from 9 brains); *dfmr1³* (n=74 cells from 11 brains); Figs. 5C and 5D). These differences were not detected in intact brains, perhaps owing to heightened sensitivity at single cell resolution. However, over the longer period of maintained depolarization, Ca^{2+} dynamics in mature cultures closely mimicked whole brain MB imaging (compare Figs. 4C,D and 5C,D). As in the intact MB, there was a >60% increase in Ca^{2+} reporter fluorescence intensity in *dfmr1^{50M}* null MB Kenyon cells compared to control neurons during many minutes of maintained depolarization (Fig. 5C; p values from 0.03–0.01) which was even more pronounced in the *dfmr1³* allele (Fig 5D; p values from 0.01–0.001). Therefore, both in whole brain MBs and in dissociated primary culture MB Kenyon cells, *dfmr1* null neurons exhibit altered Ca^{2+} dynamics following release from internal stores, with an elevated accumulation of cytosolic Ca^{2+} following depolarization.

We next examined the release of Ca^{2+} from stores in MB Kenyon cells expressing dFMRP in the *dfmr1^{50M}* null background (Supplemental Fig. 1). This resulted in a complete rescue of the elevated calcium release defect of the null (Fig. 5E). Both control and dFMR1 cultures reached a maximum of fluorescence increase after membrane depolarization of ~20% over baseline values (p values from 0.13–0.7, control (n=39 cells from 10 brains); dFMR1 (n=80 cells from 12 brains)). Thus, in MB Kenyon cell cultures, introduction of wildtype dFMRP is able to completely restore calcium release dynamics after membrane depolarization.

dFMRP regulates Ca^{2+} signaling fidelity in developing MB neurons

In the above analyses, the most significant Ca^{2+} dynamic defects consistently manifest in the mature MB circuit. However, our previous work identified the early post-eclosion period as the time of maximal dFMRP expression, translational misregulation and activity-dependent pruning defects in the MB circuit (Tessier and Broadie, 2008). We therefore examined more closely the Ca^{2+} transient and Ca^{2+} store release profiles at the 0–3 hour post-eclosion time point (Figs. 2–5). Interestingly, while the averages of these parameters in each experiment were not significantly dependent on dFMRP, there was a striking increase in the variability of measurements in *dfmr1^{50M}* mutants compared to controls. We therefore conclude that there is a loss of Ca^{2+} signaling fidelity during MB circuit maturation in *dfmr1^{50M}* nulls.

Immediately post-eclosion (0–3 hours), the distribution of individual Ca^{2+} transient amplitudes was significantly (p=0.02) broader in *dfmr1^{50M}* null MBs compared to controls (Fig. 2). Indeed, ~40% of *dfmr1^{50M}* brains produced Ca^{2+} transient amplitudes that were outside the normal distribution exhibited in controls. Similarly, loss of dFMRP at 0–3 hours produced a highly significant (p=0.006) increase in the distribution of the magnitude of Ca^{2+} release from internal organelle stores (Fig. 4). A population of *dfmr1^{50M}* MB neurons (~20%) exhibited a large elevation in Ca^{2+} cytosolic accumulation levels, which was never observed in controls. Thus, while statistically significant changes do not occur when comparing mean values, there are populations of *dfmr1* mutant animals that deviate significantly from wildtype. This loss of fidelity in regulating these two aspects of calcium homeostasis is consistent with findings in the mouse FXS model which identified inconsistent firing of calcium spikes after stimulation (Meredith et al., 2007). These data are also consistent with the large variability in FXS phenotypes across the affected human population (Fryns et al., 1984; Rogers et al., 2001; Sabaratnam et al., 2001; Torrioli et al., 2008). The early loss of Ca^{2+} control in the developing MB circuit may set the stage for the more reproducible changes in Ca^{2+} dynamics evident later in life.

dFMRP regulates mRNA expression of Ca^{2+} buffering proteins

Regulation of calcium homeostasis in neurons involves a complex array of transporters, channels, pumps, calcium sensors and calcium binding proteins (Hardie and Minke, 1993; Parekh and Putney, 2005; Saimi and Kung, 1994). In an effort to begin to understand the

molecular players that may be involved in the dFMRP-dependent processes of calcium signaling, we investigated the expression of major *Drosophila* calcium sensors and calcium buffering proteins. Given the diversity of mechanisms involved in potentially regulating both calcium transients and release of calcium from stores, it seemed reasonable that calcium-binding proteins may be involved in both processes.

Two major *Drosophila* Ca²⁺-binding proteins, frequenin 1 and 2, are the founding members of mammalian neuronal calcium sensor-1 class of proteins (Hilfiker, 2003). Frequenins are high-affinity Ca²⁺ binding proteins whose over-expression or loss of function result in dramatic changes in the Ca²⁺ signaling regulating synaptic transmission (Pongs et al., 1993; Rivoecchi et al., 1994; Romero-Pozuelo et al., 2007; Sanchez-Gracia et al., 2010). Loss-of-function mutations in the Ca²⁺-binding protein calmodulin similarly result in elevated neurotransmission (Arredondo et al., 1998). In addition, the Ca²⁺ buffering protein calbindin also regulates Ca²⁺ dynamics to modify neuronal signaling (Barski et al., 2003; Reifegerste et al., 1993). In mammals, genetic ablation of calbindin results in increased Ca²⁺ transients amplitudes in Purkinje parallel and climbing fibers (Barski et al., 2003). We therefore performed quantitative RT-PCR for these critical Ca²⁺-binding proteins on RNA extracted from heads of control and *dfmr1* null animals at both 0–3 hours and 4 days of age.

All of these Ca²⁺-binding proteins showed changes in mRNA expression over developmental time that paralleled previous observations in MB Ca²⁺ levels (Fig. 1) and dFMRP-dependent Ca²⁺ dynamics (Fig. 2 and 4). At the immature 0–3 hour time point, frequenin 2 mRNA expression was approximately 1.3 fold increased (relative values: control=1, *dfmr1*^{50M}=1.3, n≥4 replicates/3 experiments, p=0.01) in *dfmr1*^{50M} mutants compared to controls (Fig. 6). In contrast, frequenin 1 showed no detectable change (relative values: control=1, *dfmr1*^{50M}=0.99). Interestingly, the expression in *dfmr1* null mutants of calbindin was only half that of controls at this immature time point (relative values: control=1, *dfmr1*^{50M}=0.5, n≥4 replicates/3 experiments, p<0.001), whereas calmodulin showed more minimally reduced expression (relative values: control=1, *dfmr1*^{50M}=0.9, p<0.01; Fig. 6). By 4 days of age, however, all Ca²⁺-binding proteins showed reduced mRNA expression, with calmodulin and calbindin showing ~40% (relative values: control=1, *dfmr1*^{50M}=0.6, n≥4 replicates/3 experiments; p<0.001) and ~50% (relative values: control=1, *dfmr1*^{50M}=0.5; p=0.01 n≥4 replicates/3 experiments; p<0.001) reductions in *dfmr1* nulls, respectively (Fig. 6). The Ca²⁺ sensors frequenin 1 and 2 showed milder phenotypes of ~25% (relative values: control=1, *dfmr1*^{50M}=0.73, n≥4 replicates/3 experiments; p<0.001) and ~15% (relative values: control=1, *dfmr1*^{50M}=0.86; p=0.01) reductions in *dfmr1* nulls, respectively (Fig. 6). These data suggest that complex regulation of Ca²⁺-binding proteins occurs in development, which may be the result of the high dFMRP-dependent variability in neuronal activity present during the post-eclosion brain refinement period. Reduced Ca²⁺ buffering protein expression, and thus reduced buffering capacity, is consistent with the augmented strength of calcium signaling in *dfmr1* null MBs.

Discussion

Fragile X syndrome (FXS) patients suffer an array of activity-related symptoms including hyperactivity, hypersensitivity to sensory stimuli and childhood susceptibility to seizures, which suggest heightened activity at the cellular or neural circuit levels that may be developmentally modulated (Fryns et al., 1984; Hagerman and Stafstrom, 2009; Sabaratnam et al., 2001; Torrioli et al., 2008). Consistently, FXS animal models robustly display hyperactivity and audiogenic-induced seizures, and have defects in activity-regulated processes of neuronal and circuit function, many of which are developmentally modulated (Bushey et al., 2009; Galvez and Greenough, 2005; Irwin et al., 2002; Pacey et al., 2009; Pan et al., 2008; Tessier and Broadie, 2008; Yun et al., 2006). Moreover, molecular phenotypes

associated with loss of FMRP are commonly revealed under conditions of synaptic stimulation or specific neurotransmitter receptor activation (Antar et al., 2004; Gabel et al., 2004; Huber et al., 2002; Park et al., 2008; Repicky and Broadie, 2009; Todd et al., 2003; Valentine et al., 2000; Zalfa et al., 2003). One possible bidirectional link between FMRP function and neuronal activity is the regulation of cytosolic calcium levels, which undergo dramatic homeostatic changes during development and continually in response to neuronal firing (Babai et al., 2009; Berridge et al., 2000; Kuromi et al., 2004; Lohmann, 2009). Using a transgenic fluorescent calcium reporter to monitor changes in calcium dynamics after neuronal depolarization, we have characterized developmentally modulated defects in calcium regulation that may underlie learning and memory deficits in the *Drosophila* FXS model.

The *Drosophila* Mushroom Body (MB) is a well-characterized learning and memory center, and learning and memory defects in the *Drosophila* FXS model map to this circuit (Blum et al., 2009; Bolduc et al., 2008; Dubnau et al., 2001; Heisenberg et al., 1985; McBride et al., 1999; McGuire et al., 2001). To probe possible roles of dFMRP in MB calcium signaling, we first analyzed basal calcium levels in *dfmr1* nulls relative to controls. All genotypes show a dramatic increase in MB calcium levels between the immature time point of 0–3 hours post-eclosion and the mature time point of 4 days post-eclosion. Although there are no significant differences between *dfmr1* mutants and controls at either time, the rate of the calcium accumulation during MB maturation is twice as great in wildtype compared to the *dfmr1* null. It is unclear why the MB exhibits increased cytosolic calcium in the adult, but it is notable that rat hippocampal neurons similarly show an age-dependent increase in resting calcium concentrations (Raza et al., 2007). One possibility is that the requirement for calcium in developing and refining synaptic connections is reduced relative to that required for maintaining proper connections and responding to synaptic plasticity demands. To some degree, this does seem to be the case at the level of dendritic spine development in mammalian neurons, as low calcium concentrations promote maturation of spine structures (Segal, 2001). Thus, the altered calcium profile during MB maturation might contribute to defects in MB circuitry in the absence of dFMRP, including changes in the stability and maintenance of synaptic connections (Tessier and Broadie, 2008).

Immediately after eclosion is a time of intense neural activity as the fly begins to experience its environment and needs to respond to external stimuli for the first time. This activity is required for the elimination of improper synaptic connections in the MB. Both neuronal activity and dFMRP are required for the use-dependent refinement process (Tessier and Broadie, 2008). At this developmental time point, calcium signaling in the *dfmr1* null MB is characterized by a disruption of reproducibility, manifested in a significant loss of response fidelity, albeit without statistically significant differences in mean amplitude or rise to peak times. For both depolarization-induced calcium transients and calcium release from internal stores, there were clear populations of *dfmr1* MBs that responded outside the normal distributions seen in wildtype controls. This increase in variability is not likely due to known MB midline crossing-over structural defects associated with loss of *dfmr1*, as this defect was rare in our data sets, and animals showing significant crossing-over were not included for quantification. Rather, high variability seems to be a hallmark of the FXS disease state. For example, in mouse FXS knockout models, loss of FMRP alters the frequency of synaptic connectivity and moreover results in an increased failure of dendritic spines to fire calcium spike transients (Hanson and Madison, 2007; Meredith et al., 2007). Reproducible and reliable calcium signaling is required for the proper development of nervous system circuitry (Berridge et al., 2000; Lohmann, 2009). Therefore, the reduced fidelity of calcium regulation in *dfmr1* mutants during the critical period of activity-dependent circuit refinement may result in an inappropriate neural framework, which remains susceptible to epigenetic factors. Thus, the effect on mature calcium signaling mechanisms may be an indirect consequence of the loss of dFMRP, and more dependent on global changes to synaptic regulation that occur over time.

At maturity, several calcium-signaling defects are evident in *dfmr1* null MBs. The amplitude of depolarization-induced calcium influx transients is elevated in mutant MB neurons. This defect is also significant in isolated MB neurons, and therefore does not result from MB overgrowth phenotypes (McBride et al., 2005; Michel et al., 2004; Pan et al., 2004; Tessier and Broadie, 2008). Analysis of calcium transients in *dfmr1* null primary MB neuronal culture show similar developmental control and similar amplitude elevations to defects characterized in the intact MB circuit in the brain. In addition to the calcium peak being higher, the rise to peak times are slower specifically in the *dfmr1*^{50M} mutant, resulting in an extended calcium signal time course. This defect appears specific to this mutant allele, but nevertheless, either the rate of calcium entry into the cell or the accumulation of calcium in the cytosol is significantly slowed in the absence of dFMRP in this background. It is not clear whether these two aspects of the signaling defects are related or independent. Additionally, calcium release from intracellular organelles is significantly augmented in mature *dfmr1* null MB neurons (Banerjee et al., 2006; Parekh and Putney, 2005; Ryglewski et al., 2007). The same defect is manifested in primary culture, where excessive release of calcium from mutant neurons is detectable in both immature and mature cultures. Interestingly, the developmental stage of cultured neurons alters their capacity to process calcium from stores after depolarization. There is an overall reduced capacity of immature cultured neurons (3 DIV) to release stored calcium after depolarization. After 15 minutes of depolarization, the total fluorescence changes were approximately 60% lower than those seen in more mature cultured neurons (6 DIV). It is possible that this difference may be linked to the reduced number of processes and the lack of synaptic connections in these immature neurons at this early developmental stage (Oh et al., 2008). The larger release of calcium at 6 DIV may be due to a large contribution from synaptic mechanisms not present at 3 DIV. Similarly, the augmented release from *dfmr1* null neurons could either arise from an increase in calcium efflux from storage organelles or a change in the calcium buffering/retention properties of the neuronal/synaptic cytosol. In either case, the dysregulation of calcium homeostasis in *dfmr1* mutants is clearly a wide-ranging effect impacting multiple sources of calcium (external and organelle stores) and, perhaps, owing to multiple molecular defects.

The calcium dynamic defects we identified in the null mutants were modulated by transgenic introduction of wildtype dFMRP, both in the intact brain and in dissociated neuronal cultures. We co-expressed the calcium reporter and dFMRP with Mushroom Body OK107-GAL4 in *dfmr1*^{50M} null brains, which resulted in dFMRP overexpression. This overexpression is likely due to the fact that the endogenous dFMRP level normally declines as the animal matures, whereas the OK107 driver controlling ectopic expression is not regulated in the same manner (Tessier and Broadie, 2008; Wang et al., 2004a). Overexpression of dFMRP augmented calcium transients in the brain and in culture. Precedent for these phenotypes is found at the larval neuromusculature junction (NMJ), where excitatory junctional currents (EJCs) produced by neuronal overexpression of dFMRP mimic the null condition, whereas miniature EJC frequency and amplitude are significantly augmented by the same genetic condition (Zhang et al., 2001). In addition, recent attempts to reverse dendritic spine defects in the mouse FXS model with pharmacologies typically able to rescue loss of FMRP phenotypes, similarly exacerbate immature spine hallmarks in FMR1 null mice (Cruz-Martin et al., 2010). Thus, the cellular context in which the transients are measured may be critical in ascertaining dFMRP function. In the whole brain, dFMRP was expressed exclusively in the presynaptic axon which may highlight a direct role in regulating calcium entry. Conversely, in culture, there were no structural or cell-to-cell contact criteria used to select cells for quantitative analysis. Thus, elevated transients in culture would be predicted to be more apparent if only GFP positive cells (dFMRP overexpressing cells) that synapse onto non-GFP (*dfmr1* null) cells were considered. Alternatively, calcium transients assayed in cell soma may exhibit significantly different regulation than synapse-rich axonal processes.

Targeted dFMRP expression also altered the *dfmr1* null defects in calcium release from internal stores. In this condition, calcium store release was significantly reduced in the context of the whole brain MB circuit, and completely rescued the excessive store release of *dfmr1* null MB neurons in culture. Many molecular, cellular and behavioral defects associated with loss of FMRP are inverted upon overexpression of FMRP (Berry-Kravis and Ciurlionis, 1998; Pan et al., 2004; Peier et al., 2000; Tessier and Broadie, 2008). Other FMR1 null defects, such as enhanced hippocampal long term depression and G-protein receptor coupled kinase expression in the prefrontal cortex, are completely rescued by FMRP overexpression (Hou et al., 2006; Wang et al., 2008a). Thus, the precise level of FMRP expression can result in differential phenotypes, dependent on the context. Precisely mimicking endogenous dFMRP developmental expression will be necessary in the future to differentiate between dFMRP requirements and the consequence of dFMRP misexpression.

The *dfmr1* calcium signaling defects could arise from a number of problems. For example, the process of calcium entry may be altered due to a changed capacity to detect membrane stimulation events, altered calcium channel function or, downstream, due to a change in cytosolic calcium buffering capacity (Cornelisse et al., 2007; Egger and Stroh, 2009; Hildebrand et al., 2009; Zhang and Linden, 2009). Indeed, membrane-associated Gq signaling and the surface expression of glutamate-gated receptor channels are both altered in *dfmr1* null neurons (Gatto and Broadie, 2009; Pan et al., 2008; Repicky and Broadie, 2009; Tessier and Broadie, 2009). Nevertheless, a particularly attractive possibility is impaired calcium buffering. Several calcium-binding proteins have reduced brain expression in *dfmr1* mutants, with the critical calcium buffers calmodulin and calbindin showing the largest reduction. A reduced ability to bind calcium would be consistent with an increase in transient amplitudes, as more of the calcium influx that might normally be soaked up by buffering proteins would be visible to the gCAMP reporter. Consistent with this interpretation, mutations in both calmodulin and calbindin result in increased amplitude of calcium transients and evoked currents (Arredondo et al., 1998; Barski et al., 2003). The electrophysiological defects closely resemble phenotypes identified at the *dfmr1* null neuromuscular junction (Zhang et al., 2001). The reduced expression of these important modulators of calcium homeostasis could also explain the increase in *dfmr1* neuronal activity (Repicky and Broadie, 2009).

The *dfmr1* mutant defect in calcium store release is also consistent with the reduced expression of calcium buffering proteins. As calcium is released from organelle stores, there are fewer binding proteins present in the cytosol to limit the calcium exposed to the gCAMP reporter, resulting in a higher fluorescence signal. Consistent with the specific role for calmodulin, FMRP activation downstream of metabotropic glutamate receptor signaling requires calmodulin-dependent kinases (Muddashetty et al., 2007; Wang et al., 2008b). If FMRP directly regulates calmodulin, then amplification of neuronal signaling could be envisioned whereby FMRP upregulates calmodulin to further facilitate its own activation, or other calmodulin-dependent processes. Both mammalian and *Drosophila* FMRP are able alter global and specific mRNA levels, though it is unclear whether the mRNA reductions identified here are direct or indirect effects of loss of dFMRP (D'Hulst et al., 2006; Epstein et al., 2009; Tervonen et al., 2005; Tessier and Broadie, 2008; Xu et al., 2004; Zalfa et al., 2007). It will therefore be necessary in the future to determine whether our findings are also apparent at the protein level, and whether spatial distributions of these proteins may be important for their function in the MB, as has been demonstrated in the visual system (Porter et al., 1993). Future studies will focus on the molecular and cellular analysis of how these regulators of calcium dynamics function specifically in the MB circuit, to shed light on their relative contributions to learning and memory defects in the *Drosophila* FXS model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Developmental changes in Ca^{2+} levels altered in *dfmr1* null brain MB

A) Representative images of gCAMP calcium reporter fluorescence from control and *dfmr1^{50M}* null MBs at 1) 0–3 hours post-eclosion and 2) 4 days post-eclosion. Scale bar=20 μm . **B)** Quantification of fluorescence intensity for each genotype at each time point (0–3 hour: control n=13; *dfmr1^{50M}* n=13 and 4 days: control n=13, *dfmr1^{50M}* n=11). Significance: $0.01 > p > 0.001$ (**), $0.001 > p$ (***). Bars show mean $\pm$ SEM. **C)** Anti-GFP immunocytochemistry in control and *dfmr1* null MBs expressing the gCAMP (GFP) reporter at 0–3 hours and 4 days post-eclosion. Scale bar=20 μm . **D)** Quantification of anti-GFP fluorescence intensity for each genotype at each time point (0–3 hour: control n=12; *dfmr1^{50M}* n=12; 4 days: control n=17; *dfmr1^{50M}* n=12). Bars show mean $\pm$ SEM.

Figure 2. dFMRP regulates the magnitude and kinetics of MB Ca^{2+} transients

A) Representative MB images of the gCAMP calcium reporter before stimulation (left), during depolarization-induced Ca^{2+} peak (middle) and after transient peak (right). Images are pseudocolored. Scale bar=20 μm . **B)** Representative trace of the change in Ca^{2+} fluorescence relative to baseline during a transient depolarizing stimulus. Arrow indicates the time of addition of 70mM KCl. The areas of the curve used to quantify transient amplitude and rise to peak times are indicated. Quantification of Ca^{2+} transient amplitudes (**C**) and rise times to peak (**D**) in control and *dfmr1^{50M}* null MBs at 0–3 hours (control n=19, *dfmr1^{50M}* n=20) and control, *dfmr1^{50M}* and *dfmr1³* MBs at 4 days post-eclosion (control n=22, *dfmr1^{50M}* n=21, *dfmr1³* n=24). Significance: p<0.05 (*), p<0.001 (***). Bars show mean $\pm$ SEM.

Figure 3. dFMRP regulates Ca²⁺ transient amplitude in MB primary culture

A) Representative pseudocolored images of MB Kenyon cells expressing gCAMP calcium reporter in primary neuronal cultures before stimulation (Nomarski, left panel), during transient 70mM KCl depolarization (middle panel), and after transient peak (right). Scale bar=5μm.

B) Representative trace of the change in Ca²⁺ fluorescence relative to baseline during a transient depolarizing stimulus. Arrow indicates the time of addition of 70mM KCl. The differential used to quantify transient amplitude is indicated.

C) Quantification of Ca²⁺ transient amplitudes from control and *dfmr1*^{50M} null cells grown for 3 days *in vitro* (control n=68 cell from 10 brains, *dfmr1*^{50M} n=67 cells from 10 brains) and control, *dfmr1*^{50M} and *dfmr1*³ cells grown for 6 days *in vitro* (control n=49 cells from 10 brains, *dfmr1*^{50M} n=44 cells from 7 brains, *dfmr1*³ n=72 cells from 11 brains). Significance: 0.001<p<0.01 (**), p<0.001 (***). Bars show mean ± SEM.

Figure 4. dFMRP regulates Ca^{2+} release from internal stores in MB circuit

A) Representative pseudocolored images of MB expressing the gCAMP calcium reporter before stimulation (left panel) and 5 and 15 minutes after depolarizing stimulation (middle and right panels, respectively). Arrows indicate regions of increasing fluorescence over time. Scale bar=20 μm . **B)** Quantification of fluorescence intensity after stimulation from 0–3 hour post-eclosion control (n=20) and *dfmr1^{50M}* (n=25) animals. **C)** Quantification of fluorescence intensity after stimulation from 4 day post-eclosion control (n=20) and *dfmr1^{50M}* (n=19) animals. **D)** Quantification of fluorescence intensity after stimulation from 4 day post-eclosion control (n=20) and *dfmr1³* (n=21) animals. **E)** Quantification of fluorescence intensity after stimulation from 4 day post-eclosion control (n=20) and UAS-*dfmr1*; OK107-GAL4 (dFMR1; n=20) animals. Significance: 0.05>p>0.01 (*), 0.01>p>0.001 (**). Bars show mean ± SEM.

Figure 5. dFMRP regulates Ca^{2+} release from internal stores in primary culture

A) Representative images of gCAMP calcium reporter in MB Kenyon cells in brain primary neuronal cultures before depolarizing stimulation (Nomarski, left panel) and then 5 and 15 minutes after stimulation (pseudocolored middle and right panels, respectively). Arrows indicate cell bodies with increasing fluorescence over time. Inset shows higher magnification of a single cell from the boxed region. Scale bar=20 μm . **B)** Quantification of changes in gCAMP fluorescent intensity from MB cultures grown for 3 days *in vitro* as a function of time post-stimulation: control (n=60 cells from 10 brains) and *dfmr1^{50M}* (n=89 cells from 12 brains). **C)** Quantification of changes in gCAMP fluorescent intensity from MB cultures grown for 6 days *in vitro* as a function of time post-stimulation: control (n=39 cells from 10 brains) and *dfmr1^{50M}* (n=50 cells from 12 brains). **D)** Quantification of an independent *dfmr1* allele: control (n=39 cells from 10 brains) and *dfmr1³* (n=74 cells from 11 brains). **E)** Quantification of changes in gCAMP fluorescent intensity from MB cultures grown for 6 days *in vitro* as a function of time post-stimulation made from control (n=39 cells from 10 brains) and UAS-*dfmr1*; OK107-GAL4 (dFMR1; n=80 cells from 12 brains). Significance: 0.05>p>0.01 (*), 0.01>p>0.001 (**), p<0.001 (***). Bars show mean $\pm$ SEM.

Figure 6. dFMRP regulates mRNA expression of calcium binding proteins

Quantitative RT-PCR from RNA extracted from 0–3 hour old and 4 day old control and *dfmr1*^{50M} null brains. All values were first normalized to *gapdh2* signals and then calculated as a relative fold change of *dfmr1* to control values. Dashed line indicates values corresponding to no difference in expression between genotypes (n=3 for each genotype). Significance: 0.05>p>0.01 (*), 0.01>p>0.001 (**), 0.001>p (***) . Bars show mean ± SEM.