Neuron class-specific requirements for Fragile X Mental Retardation Protein in critical period development of calcium signaling in learning and memory circuitry

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ABSTRACT

Neural circuit optimization occurs through sensory activity-dependent mechanisms that refine synaptic connectivity and information processing during early-use developmental critical periods. Fragile X Mental Retardation Protein (FMRP), the gene product lost in Fragile X syndrome (FXS), acts as an activity sensor during critical period development, both as an RNA-binding translation regulator and channel-binding excitability regulator. Here, we employ a Drosophila FXS disease model to assay calcium signaling dynamics with a targeted transgenic GCaMP reporter during critical period development of the mushroom body (MB) learning/memory circuit. We find FMRP regulates depolarization-induced calcium signaling in a neuron-specific manner within this circuit, suppressing activity-dependent calcium transients in excitatory cholinergic MB input projection neurons and enhancing calcium signals in inhibitory GABAergic MB output neurons. Both changes are restricted to the developmental critical period and rectified at maturity. Importantly, conditional genetic (dfmr1) rescue of null mutants during the critical period corrects calcium signaling defects in both neuron classes, indicating a temporarily restricted FMRP requirement. Likewise, conditional dfmr1 knockdown (RNAi) during the critical period replicates constitutive null mutant defects in both neuron classes, confirming cell-autonomous requirements for FMRP in developmental regulation of calcium signaling dynamics. Optogenetic stimulation during the critical period enhances depolarization-induced calcium signaling in both neuron classes, but this developmental change is eliminated in dfmr1 null mutants, indicating the activity-dependent regulation requires FMRP. These results show FMRP shapes neuron class-specific calcium signaling in excitatory vs. inhibitory neurons in developing learning/memory circuitry, and that FMRP mediates activity-dependent regulation of calcium signaling specifically during the early-use critical period.

1. Introduction

Fragile X syndrome (FXS) is the leading heritable cause of intellectual disability (Boyle and Kaufmann, 2010) and most prevalent single-gene autism spectrum disorder (Wang et al., 2012). The disease state is caused by lack of Fragile X Mental Retardation Protein (FMRP), a key regulator of activity-dependent neural circuit modulation during critical period development (Doll and Broadie, 2014). The full range of FMRP function is broad and inconclusive, but includes regulation of multiple classes of voltage-gated ion channels, via both RNA-binding translational control (Gross et al., 2011; Lee et al., 2011; Strumbos et al., 2010) and direct channel-binding interactions (Brown et al., 2010; Deng et al., 2013; Ferron et al., 2014; Zhang et al., 2014). Downstream, FMRP also regulates numerous calcium-binding proteins (CaBPs) involved in activity-dependent calcium signaling (Chen et al., 2003; Tessier and Broadie, 2011; Wang et al., 2009). Consistently, FXS disease models exhibit increased excitatory neurotransmission (Deng et al., 2013; Deng et al., 2011; Gibson et al., 2008), defects in action potential termination (Brown et al., 2010; Deng et al., 2013; Zhang et al., 2012) and altered depolarization-triggered calcium influx (Deng et al., 2013; Deng et al., 2011; Ferron et al., 2014; Patel et al., 2013; Tessier and Broadie, 2011). Earlier work has elegantly mapped critical period functional refinement (Meredith, 2015), and defining FMRP roles in these activity-dependent mechanisms at single cell resolution during critical periods is now possible through the application of incisive Drosophila genetics.

The Drosophila FXS disease model has long been instrumental in dissecting developmental and activity-dependent FMRP roles (Doll and Broadie, 2015; Gatto and Broadie, 2008; Pan et al., 2004; Tessier and Broadie, 2008; Weisz et al., 2015; Zhang et al., 2001). Our past studies have identified hyper-excitability, calcium influx and store release defects, and changes in CaBP expression in the absence of FMRP function (Gatto and Broadie, 2008; Repicky and Broadie, 2009; Tessier and...
2. Materials & methods

2.1. Drosophila genetics

All stocks were reared on standard cornmeal/agar/molasses food at 25 °C unless stated otherwise. Multiple recombinant lines of dfmr1 null allele dfmr1 (Zhang et al., 2001) were generated with standard genetic techniques using a combination of the following transgenic lines; 1) R12G04-Gal4, R65G01-Gal4, 20UXA-IVS-GCamp5G and tub-Gal80(ts); TM2; TM6 from the Drosophila Stock Center (Bloomington, IN, USA), 2) UAS-dfmr1-RNAI (TRIP, Harvard) and 3) UAS-ChR2(H134R)-mCherry generously provided by Leslie Griffith (Pulver et al., 2009). For conditional Gal80ts dfmr1-RNAI and wildtype dfmr1-rescue (UAS-9557-3, wildtype dfmr1 under UAS control; Gatto and Broadie, 2009) experiments, both controls and experimental animals were raised at permissive 18 °C (Gal80ts active) until the last pupal day (P4), and then shifted to restrictive 29 °C (Gal80ts inactive) until 1 day post-eclosion (dpe).

2.2. Immunocytochemistry and imaging

Immunocytochemistry was carried out as previously described (Doll and Broadie, 2015). Primary rabbit anti-GFP (ab2990; Abcam, Cambridge, UK) was used at a 1:2000 dilution, and secondary antibody anti-mouse-IgG AlexaFlour 488 (Molecular Probes, Eugene, OR) was used at a 1:500 dilution. Images were acquired for the central brain region on a Zeiss Meta 510 confocal microscope with 40–100× objectives, and collected as Z-stacks of 1 μm section depth.

2.3. Calcium imaging

The 20UXA-IVS-GCamp5G line (Bloomington stock center #42037) was used as a transgenic [Ca^{2+}] reporter (Akerboom et al., 2012). This reporter was crossed to R12G04-Gal4 and R65G01-Gal4 driver lines in both w^{1118} genetic background control and dfmr1 null mutant backgrounds. Acutely dissected brains from all four genotypes were immobilized on 30 mm petri dishes in 3 mL of physiological saline containing 128 mM NaCl, 2 mM KCl, 4 mM MgCl2, 35.5 mM sucrose, 5 mM Hepes, and 1.8 mM Ca^{2+}, pH 7.2 (Tessier and Broadie, 2011). Labeled cell soma were immediately imaged with a 40× water-immersion objective with maximal pinhole aperture on a Zeiss LSM510Meta laser-scanning confocal microscope. Each neuron was outlined using a region of interest (ROI) box, and a time series was captured at 128 × 128 resolution at maximum scanning speeds (44 ms/frame). Baseline fluorescence was determined for each selected neuron (~250 frames/15 s), followed by acute K+ depolarization (60 mM KCl) (Gatto et al., 2014) with recording for 2500 total frames (110 s). Image was used for image registration and determination of baseline fluorescence intensity values within the ROI (Schneider et al., 2012). Baseline fluorescence was defined from a 20-frame average that occurred in the 5 s prior to the onset of transient initiation and was used to normalize the data set. Data were plotted as the relative change in fluorescence from baseline divided by the baseline (ΔF/F = F2 − F baseline) / F baseline), with each data point representing an average and standard error of 20 frames. Line graph data points represent an average of 20 frames (captured at 44 ms/frame), therefore every data point represents 880 ms. Only one calcium transient was captured from each individual neuron, and only one neuron was analyzed from each animal. Time to peak [Ca^{2+}] was calculated by averaging raw individual trace data, locating the frame with the greatest fluorescence value in each trial, relative to the initiation of the transient (in seconds: frame # × 0.0044 s). Average peak fluorescence values compare the maximum 20 frame fluorescent values from each group of transient initiation-aligned traces. Only individual traces with half-life decay functions in which r^2 > 0.9 were included in decay analyses. Decay half-life (t_{1/2}) was estimated using the least-squares method to fit the 20% to 80% decay fluorescence to a single-exponential function Y = Y_0 e^{-kt} where t_{1/2} = ln2 / k in MATLAB (version R2012b, Mathworks, Natick, MA).

2.4. Optogenetics

Transgenic animals were generated from pairwise crosses between the UAS-ChR2(H134R)-mCherry optogenetic channel line and 1 of 2 drivers (R12G04-Gal4 or R65G01-Gal4), both in w^{1118} genetic background control and dfmr1 null mutant backgrounds. Offspring from all four genotypes were fed from hatching on standard food supplemented with either 10 μL ETOH (in 10 mL volume; vehicle control) or 100 μM alltrans retinal (ATR), an essential co-factor for channelrhodopsin function (Ataman et al., 2008). Upon eclosion, developmentally staged animals were placed in 30 mm petri dishes with Whatman paper strips saturated in a 20% sucrose solution containing either the vehicle control or 100 μM ATR. All control and experimental animals were placed in a...
LED exposure chamber with two 470 nm blue light Luxeon Rebel Endor Star 3X 15-Watt LED arrays (LED Supply, Randolph, VT). At 15 V, the LED arrays generate ~100 μW/cm² of blue light at the working distance of 2 cm. Animals were exposed to 24 h of 20 ms light pulses at 5 Hz frequency. Brains from light-exposed animals then underwent Ca²⁺ imaging as described above.

2.5. Statistics

All statistical analyses were performed using Instat or Prism (GraphPad Software, San Diego, CA). Data from two group comparisons were analyzed with a two-tailed unpaired t-test. Data from three group or more comparisons were analyzed with one-way analysis of variance (ANOVA) and Dunnett post hoc tests were applied to compare individual experimental groups to control (F and p values reported). Transient initiation-aligned traces (mean ± standard error) for each genotype are presented in line graphs. Data are presented in box-and-whisker plots (minimum, median, maximum and quartiles). Sample size definitions for peak amplitude, time to peak amplitude and decay half-life, n = number of individual transient recordings (1 transient per neuron, per animal). Significance levels in figures are represented as p > 0.05 (not significant, n.s.), p < 0.05 (*), p < 0.01 (**) and p < 0.001 (**).
mPN2), allowing single-cell resolution recording and manipulation (Fig. 1B; Doll and Broadie, 2015; Jenett et al., 2012). mPN2 cell bodies are located at the ventrolateral edge of the central brain in the subesophageal zone (SEZ; Ito et al., 2014), with a primary process that travels medially before bifurcation into a collateral and a dorsal branch (Fig. 1B; Tanaka et al., 2012). mPN2 is characterized as a bilateral uniglomerular medial antennal lobe tract (mALT) projection neuron with a dendritic arbor specific to the VL1 glomerulus of the antennal lobe (Al; Fig. 1B2). The axon travels through the mALT, with primary presynaptic outputs to 3–5 microglomeruli in the MB calyx on Kenyon Cells (KCs) as well as further axon terminals in the lateral horn (LH; Fig. 1B3).

The R12G04-Gal4 (Oli gene) FlyLight driver specifically targets the inhibitory GABAergic output neuron MBON-γ1pc>α/s (abbreviated MBON-11; Aso et al., 2014b), formerly identified as MB-MVP2 (Fig. 1B; Aso et al., 2014b; Doll and Broadie, 2015; Jenett et al., 2012). Single MBON-11 neuronal cell bodies are located in the inferior medial protocerebrum (IPm) in the anterior portion of the central brain, with a prominent dendritic arbor within the MB spur (Fig. 1B1), and an output axon that bifurcates into collateral and vertical branches (Tanaka et al., 2008). These GABAergic MB output neurons are required in multiple sensory modalities (both olfactory and visual) for aversive memory consolidation (Aso et al., 2014b). Our previous work established a tight temporal requirement for FMRP function during the early sensory input critical period in the activity-dependent development of both mPN2 and MBON-11 dendritic arborization (Doll and Broadie, 2015).

3.2. Excitatory mPN2 displays an FMRP-dependent developmental shift in Ca\(^{2+}\) dynamics

The FMRP-dependent refinement of MB circuitry is confined to a transient window of development during early sensory use, immediately following eclosion (Doll and Broadie, 2015; Tessier and Broadie, 2008), similar to a comparable requirement in mammals (Portera-Cailliau, 2012). Based on our previous studies, we focused analyses on the 1 dpe critical period compared to the 7 dpe time point representing adult maturity. The GCamp5G transgenic [Ca\(^{2+}\)] reporter (Akerboom et al., 2012) was used to assay activity-dependent Ca\(^{2+}\) signaling dynamics in single, individually identified mPN2 and MBON-11 neuronal soma as a function of development time in \(w^{1118}\) genetic background control compared to \(dfmr1\) null mutants. Neurons were imaged to establish baseline GCamp5G fluorescence (1.8 mM extracellular bath [Ca\(^{2+}\)]+) and then acutely depolarized with high [K\(^{+}\)] (60 mM KCl) to generate optically measurable Ca\(^{2+}\) transients (Fiala and Spall, 2003). Three primary parameters were used to measure the Ca\(^{2+}\) transients: time to peak fluorescence (s), peak fluorescence intensity and the half-life of transient termination (s).

A summary of excitatory mPN2 neuron Ca\(^{2+}\) signaling dynamics at the 1 dpe critical period and at 7 dpe maturity is shown in Fig. 2. At 1 dpe, UAS-GCamp5G driven by R65G01-Gal4 provides clear fluorescent Ca\(^{2+}\) reporter transients upon acute K\(^{+}\) depolarization, which display a rapid rise to peak and slower decay (Fig. 2A). Compared to genetic background controls, \(dfmr1\) null mPN2 neurons exhibit a > 35% increase in the Ca\(^{2+}\) transient peak (control, 0.29 ± 0.04, \(n = 19\) neurons; \(dfmr1\), 0.46 ± 0.07, \(n = 17\) neurons, \(t\)-test, \(p = 1.25E - 20\); Fig. 2A). Despite this greatly increased peak amplitude, the time to peak is comparable between genotypes (control, 9.16 ± 0.98 s, \(n = 19\); \(dfmr1\), 10.61 ± 1.20 s, \(n = 17\), \(t\)-test, \(p = 0.35\)). Similarly, both wildtype and \(dfmr1\) null mPN2 neurons display comparable slow Ca\(^{2+}\) transient decay properties (control \(t_{1/2}; 37.44 ± 5.52\) s, \(r^2 = 0.96 ± 0.01, n = 19\); \(dfmr1\) \(t_{1/2}; 40.82 ± 6.82\), \(r^2 = 0.96 ± 0.01, n = 17, p = 0.70\); Fig. 2A, bottom). These data show that FMRP limits the level of depolarization-induced Ca\(^{2+}\) signaling in mPN2 neurons, but does not impact the rate of Ca\(^{2+}\) clearance. Therefore, the extended calcium signaling profile in \(dfmr1\) nulls (extended return to baseline; Fig. 2A) is likely rooted in elevated influx capacity and not decay properties.

At maturity (7 dpe), depolarization-induced Ca\(^{2+}\) transients in excitatory mPN2 neurons have increased dramatically in wildtype (compare Fig. 2A and B). In contrast, the developmental change in \(dfmr1\) null mutants is blunted, with a much smaller increase in Ca\(^{2+}\) transient amplitude. As a result, depolarization-induced Ca\(^{2+}\) transients at 7 dpe are largely indistinguishable between wildtype and \(dfmr1\) null mPN2 neurons (Fig. 2B). Interestingly, at maturity the peak fluorescent amplitude is actually moderately reduced (−19%) in \(dfmr1\) nulls (0.69 ± 0.10,
n = 18 neurons) compared to controls (0.85 ± 0.08, n = 22 neurons, t-test, p = 1.02E–08; Fig. 2B), underscoring the dramatic developmental shift in wildtype Ca\(^{2+}\) signaling dynamics. The temporal Ca\(^{2+}\) transient characteristics in both control and mutant genotypes are comparable at maturity, including time to peak (control, 6.77 ± 0.55 s, n = 22; dfmr1, 7.57 ± 0.45 s, n = 18, t-test, p = 0.29, n.s.; Fig. 2B), and decay half-life (control t\(_{1/2}\), 20.23 ± 2.85 s, r\(^2\) = 0.97 ± 0.02, n = 20; dfmr1 t\(_{1/2}\), 33.89 ± 8.85, r\(^2\) = 0.92 ± 0.02, n = 17, t-test, p = 0.13, n.s.; Fig. 2B). These results suggest a restricted critical period requirement for FMRP in depolarization-induced Ca\(^{2+}\) signaling, with wildtype undergoing a dramatic developmental shift compared to dfmr1 null mPN2 neurons, establishing comparable functional properties at maturity.

### 3.3. Inhibitory MBON-11 neurons show the opposite developmental shift in Ca\(^{2+}\) dynamics

Despite similar dendritic arbor architectural phenotypes, excitatory mPN2 and inhibitory MBON-11 neurons exhibit opposite FMRP-dependent responses to activity during critical period development (Doll and Broadie, 2015). Consistently, the functional Ca\(^{2+}\) signaling dynamics of these two neuron classes also display an opposite critical period-specific shift in dfmr1 mutants. At the 1 dpe critical period, acute depolarization of wildtype MBON-11 neurons causes a rapid rise to peak (Ca\(^{2+}\)\(_{\text{peak}}\)) and a quick decay back to baseline (Fig. 3A). In dfmr1 mutants, however, the 1 dpe Ca\(^{2+}\) transient peak is very significantly reduced in MBON-11 neurons (0.71 ± 0.08, n = 20 neurons) compared to controls (1.29 ± 0.14, n = 15 neurons, t-test, p = 3.95E–53; Fig. 3A). Moreover, the time to peak is significantly longer in dfmr1 null neurons (9.81 ± 0.89 s, n = 15) compared to controls (6.28 ± 0.80 s, n = 20, t-test, p = 0.0077; Fig. 3A, bottom), although the Ca\(^{2+}\) transient decay half-life is comparable between genotypes (control t\(_{1/2}\), 7.22 ± 1.8, r\(^2\) = 0.95 ± 0.01, n = 20; null t\(_{1/2}\), 9.07 ± 1.4 s, r\(^2\) = 0.94 ± 0.01, n = 15, t-test, p = 0.41, n.s.). The critical period reduction in Ca\(^{2+}\) transients in dfmr1 null MBON-11 GABAergic neurons contrasts sharply with the elevation in dfmr1 null mPN2 cholinergic neurons, showing a clear distinction between inhibitory and excitatory neurons in the developing MB circuit.

At maturity (7 dpe), wildtype MBON-11 neurons show a clear shift in Ca\(^{2+}\) transient peak fluorescence (0.81 ± 0.11, n = 22 neurons), as they undergo a prominent decrease from the developmental critical period to adulthood (compare Fig. 3A and B). In contrast to the genetic background control, dfmr1 null MBON-11 neurons retain remarkably similar activity-dependent Ca\(^{2+}\) signaling dynamics at maturity compared to the 1 dpe critical period (compare Fig. 3A and B). As in mPN2 neurons, the dfmr1 null peak transient amplitude (0.83 ± 0.10, n = 23 neurons, t-test: p = 0.55) and time to peak (6.40 ± 0.57 s, n = 23, t-test: p = 0.81) in inhibitory MBON-11 neurons were both indistinguishable from controls at maturity (Fig. 3B). The only dfmr1 phenotype remaining at maturity is a significant increase in decay half-life compared to controls (5.88 ± 0.48 s, r\(^2\) = 0.97 ± 0.01, n = 20, t-test: p = 0.0266). Taken together, these data show reduced critical period Ca\(^{2+}\) signaling in FXS disease state inhibitory MB output neurons, another transient critical period FMRP requirement that is nearly completely resolved at maturity.

### 3.4. Critical period conditional control of FMRP in excitatory mPN2 neurons

Conditional control of gene function is a powerful method for developmental dissection of neural circuit formation (Bohm et al., 2010; Fore et al., 2011) and disease mechanisms (Frickenhaus et al., 2015; Herrera et al., 2013; Liu et al., 2015; Ng and Jackson, 2015). We demonstrated above that FMRP function is crucial for regulation of Ca\(^{2+}\) dynamics in both mPN2 excitatory neurons and MBON-11 inhibitory neurons only during the early-use critical period and no longer at maturity. To test this apparently restricted FMRP requirement, we next used transgenic techniques to conditionally control FMRP expression only during the critical period and specifically within only the targeted neurons. We first used the Gal80ts repressive technique (McGuire et al., 2003; Tutor et al., 2014) to temporally restore wildtype FMRP to otherwise dfmr1 null mutants in cell-autonomous studies. In our hands, this technique provides a highly targeted method to temporally express FMRP in an otherwise completely FMRP-deficient brain (Doll and Broadie, 2015). For mPN2 neurons, this approach involved first generating and then crossing tub\(^{-}\)-Gal80ts: dfmr1\(^{50M}\) R65G01-Gal4 to UAS-GCamp5G; dfmr1\(^{50M}\), UAS-9557-3 animals. UAS-9557-3 is a genomic wildtype

![Fig. 3](image-url)
dfmr1 under UAS control. All genotypes were raised at restrictive 18 °C and then shifted to permissive 29 °C at P4, relieving Gal80ts repression and inducing dfmr1 expression only in mPN2 neurons (Fig. 4A). At the end of the critical period (1 dpe), brains were dissected and acutely depolarized (60 mM KCl) to measure Ca²⁺ transients.

Wildtype dfmr1 rescue of null phenotypes was tested by comparing three conditions (Fig. 4C): 1) dfmr1 mutants containing all transgenic elements (dfmr1SIM, R65G01-Gal4>dfmr1SIM, UAS-GCamp5G; gray line), 2) dfmr1 constitutive rescue (dfmr1SIM, R65G01-Gal4>UAS-GCamp5G; dfmr1SIM, UAS-9557-3; black line) and 3) critical period conditional rescue (tubP-Gal80ts; dfmr1SIM, R65G01-Gal4>UAS-GCamp5G; dfmr1SIM, UAS-9557-3; red line). Both constitutive (0.30 ± 0.04, n = 22 neurons, Dunnett, p < 0.001) and critical period-specific rescue (0.25 ± 0.04, n = 25 neurons, Dunnett, p < 0.001) caused a similar reduction in the peak Ca²⁺ transient compared to dfmr1 nulls (0.52 ± 0.11, n = 15 neurons, F(2,57) = 38.910, p < 0.001; Fig. 4C). Both depolarization-induced rescue transients are comparable to wildtype (compare Figs. 2 and 4C). Transgenic rescue led to no significant differences in time to peak (control, 9.74 ± 1.34 s, n = 15; constitutive rescue, 9.21 ± 0.90 s, n = 22; conditional rescue, 7.31 ± 0.67 s, n = 25; F(2,59) = 2.32, p = 0.11, n.s.) or decay half-life (control t1/2, 19.94 ± 2.21 s, r² = 0.96 ± 0.01, n = 14; constitutive rescue t1/2, 27.85 ± 3.63 s, r² = 0.96 ± 0.01, n = 20; conditional rescue t1/2, 22.53 ± 2.55 s, r² = 0.95 ± 0.01, n = 22; F(2,53) = 2.273, p = 0.19, n.s.). Thus, FMRP supplied only during the critical period completely rescues functional requirements to restore Ca²⁺ signaling properties.

To complement the wildtype rescue in mPN2 neurons, cell autonomous knockdown of dfmr1 was achieved with conditional transgenic RNA interference (RNAi). Animals were raised at the Gal80ts restrictive temperature (18 °C) and then shifted to the restrictive temperature (29 °C) at P4 to inactivate Gal80ts and induce dfmr1 RNAi (Fig. 4B). Three conditions are compared (Fig. 4D): 1) control with no RNAi or Gal80ts (R65G01-Gal4>UAS-GCamp5G; gray line), 2) constitutive dfmr1 RNAi (R65G01-Gal4>UAS-GCamp5G; dfmr1-RNAi, black line) and 3) critical period RNAi knockdown (tubP-Gal80ts; dfmr1SIM, R65G01-Gal4>UAS-GCamp5G; dfmr1SIM, UAS-9557-3; red line). Both RNAi knockdown transients are comparable to wildtype (compare Figs. 2 and 4D). Parallel, MBON-11 critical period rescue and knockdown (dfmr1 SIM, R65G01-Gal4>UAS-GCamp5G, dfmr1RNAi, black line) show similar results. Each plot represents the change of average fluorescent intensity over time (mean ± SEM), and includes an inset peak intensity histogram (minimum, median, maximum and quartiles). Significance determined by one-way ANOVA and indicated as ***p < 0.001 or not significant (n.s.).
null mutant condition, and indeed results in a stronger effect than constitutive knockdown (Fig. 4F). Temporally targeted dfmr1 knockdown only in the P4–1 dpe critical period and only in MBON-11 neurons causes a dramatic decrease in peak Ca\(^{2+}\) transient amplitude (0.41 ± 0.09, n = 10 neurons, Dunnett, p < 0.001) compared to controls (1.05 ± 0.10, n = 15 neurons; F\(_{1,23} = 40.286, p < 0.0001\); Fig. 4F). Constitutive dfmr1 knockdown of dfmr1 also causes a clear and significant reduction in peak Ca\(^{2+}\) transient amplitude (0.73 ± 0.09, n = 21 neurons, Dunnett, p < 0.001; Fig. 4F). We noted a slight difference in the relative time to peak Ca\(^{2+}\) influx in the conditional knockdown condition (control, 5.590 ± 0.835 s, n = 15 neurons; constitutive RNAi, 6.15 ± 0.64 s, n = 21, Dunnett, p > 0.05); constitutive RNAi, 9.44 ± 1.57 s, n = 10, Dunnett, p < 0.05; F\(_{2,43} = 6.935, p = 0.0025\); Fig. 4F). Finally, decay half-life was not significantly different across all groups, despite a trend toward an extended decay in the conditional knockdown (control t\(_{1/2}\), 7.20 ± 0.79 s, r\(^2\) = 0.97 ± 0.01, n = 19; constitutive RNAi t\(_{1/2}\), 6.98 ± 0.72 s, r\(^2\) = 0.95 ± 0.01, n = 25; conditional RNAi t\(_{1/2}\), 10.13 ± 1.83 s, r\(^2\) = 0.94 ± 0.01, n = 14; F\(_{2,43} = 2.205, p = 0.1199\); Fig. 4F). Thus, conditional removal of FMRP during the critical period actually provides a more robust effect than constitutive loss in MBON-11 neurons, an effect that also occurs in mPN2 neurons. Taken together, these results indicate an essential and restricted FMRP requirement in shaping activity-induced Ca\(^{2+}\) transients, specifically during the early-use critical period window of development.

3.6. Optogenetic critical period stimulation alters FMRP-dependent Ca\(^{2+}\) transients

Previous work has revealed that FMRP is required for activity-dependent changes in MB circuit architecture and synaptic connectivity during the early-use critical period (Doll and Broaddie, 2015; Tessier and Broaddie, 2008). To test whether developmental activity induces changes in Ca\(^{2+}\) signaling dynamics, we co-expressed the depolarizing UAS-ChR2 (ChR2) channel along with the UAS-GCamp5G [Ca\(^{2+}\)] reporter in both excitatory input mPN2 and inhibitory output MBON-11 neurons, exposed newly-eclosed flies to a stimulating light paradigm (470 nm illumination of 20 ms pulses at 5 Hz for 24 h) during the first day critical period following eclosion, and then performed fluorescent Ca\(^{2+}\) recordings as above. To assay mPN2 neurons, transgenic control (R65G01-Gal4–UAS-ChR2(H134R)-mCherry; UAS-GCamp5G) and dfmr1 null mutant (dfmr1\(^{GOM}\); R65G01-Gal4–UAS-ChR2(H134R)-mCherry; dfmr1\(^{GOM}\); UAS-GCamp5G) animals were raised on either EtOH vehicle or the essential ChR2 cofactor, all-trans retinal (ATR; Ataman et al., 2008; Schroll et al., 2006). ChR2 expression was verified (Fig. 5A, red) prior to GCamp5G reporter recording (Fig. 5A, green) in individually identified neurons. A summary of the results is shown in Fig. 5.

Critical period stimulation of excitatory mPN2 neurons dramatically elevates Ca\(^{2+}\) peak amplitude above vehicle control neurons (W\(_{TVA}\), 0.18 ± 0.04, n = 17 neurons; W\(_{TVA}\), 0.33 ± 0.06, n = 18 neurons, t-test, p = 0.206; 20 – 20, Fig. 5B). This amplitude change is not accompanied by a change in time to peak (W\(_{TVA}\), 9.88 ± 0.69 s, n = 17; W\(_{TVA}\), 10.43 ± 0.95 s, n = 18, t-test, p = 0.65, n.s.) or decay half-life (W\(_{TVA}\), t\(_{1/2}\), 43.99 ± 8.99 s, r\(^2\) = 0.92 ± 0.02, n = 11; W\(_{TVA}\), t\(_{1/2}\), 40.49 ± 8.10 s, r\(^2\) = 0.92 ± 0.04, n = 18, t-test, p = 0.78, n.s.). Importantly, this single cell-targeted stimulation during the critical period phenocopies Ca\(^{2+}\) transients in dfmr1 null mPN2 neurons at 1 dpe (compare to Fig. 2). Our previous work showed that dfmr1 null mPN2 neurons lack a morphological response to developmental optogenetic manipulations (Doll and Broaddie, 2015). Consistently, critical period stimulation of dfmr1 null mPN2 neurons did not significantly affect depolarization-induced Ca\(^{2+}\) peak amplitude (dfmr1\(^{GOM}\) peak amplitude, 0.41 ± 0.08, n = 18 neurons; dfmr1\(^{GOM}\), 0.38 ± 0.07, n = 14 neurons, t-test, p = 0.12, n.s.; Fig. 5C), time to peak (dfmr1\(^{GOM}\), 11.55 ± 1.00 s, n = 18; dfmr1\(^{GOM}\), 10.92 ± 1.11 s, n = 14, t-test, p = 0.68, n.s.) or half-life decay (dfmr1\(^{GOM}\) decay, 21.79 ± 2.7 s, r\(^2\) = 0.95 ± 0.02, n = 18; dfmr1\(^{GOM}\), 27.36 ± 5.72 s, r\(^2\) = 0.93 ± 0.03,
n = 13, t-test, p = 0.35, n.s.). Thus, FMRP is absolutely required for activity-dependent developmental shifts in Ca\textsuperscript{2+} signaling dynamics, providing evidence that FMRP acts as an essential activity sensor during critical period development.

We next tested the activity-dependent requirements for FMRP in shaping critical period Ca\textsuperscript{2+} signaling in inhibitory MBON-11 neurons (Fig. 6). For MBON-11, the four experimental groups were wildtype control (R12G04-Gal4\textsuperscript{N}UAS-ChR2(H134R)-mCherry; UAS-GCamp5G) and dfmr1 null (dfmr1\textsuperscript{50M}, R12G04-Gal4\textsuperscript{N}UAS-ChR2(H134R)-mCherry; dfmr1\textsuperscript{50M}, UAS-GCamp5G) raised on EtOH vehicle or ATR-supplemented food. GCamp5G (Fig. 6A, green) and ChR2 (Fig. 6A, red) expression was verified in individual MBON-11 neurons. All four genotypes were exposed to 5 Hz blue light stimulation for 24 h, with depolarization-induced Ca\textsuperscript{2+} transients assayed at the 1 dpe critical period (Fig. 6B, C). As in mPN2, stimulated MBON-11 neurons show a significant increase in peak Ca\textsuperscript{2+} transient amplitude (WT\textsuperscript{vehicle}, 0.97 ± 0.01, n = 20 neurons; WT\textsuperscript{ATR}, 1.09 ± 0.16, n = 19 neurons, t-test, p = 7.58E − 10; Fig. 6B), with no impact on time to peak (WT\textsuperscript{vehicle}, 6.70 ± 0.52 s, n = 20; WT\textsuperscript{ATR}, 6.22 ± 0.49 s, n = 19, t-test, p = 0.51, n.s.; Fig. 6B) or decay half-life (WT\textsuperscript{vehicle} t\textsubscript{1/2}, 7.36 ± 0.49 s, r\textsuperscript{2} = 0.97 ± 0.01, n = 20; WT\textsuperscript{ATR} t\textsubscript{1/2}, 8.44 ± 0.97 s, r\textsuperscript{2} = 0.95 ± 0.01, n = 19, t-test, p = 0.57, n.s.). In sharp contrast, dfmr1 null neurons are not susceptible to developmental activity modulation (compare Fig. 6B and C), as dfmr1 mutants do not exhibit any activity-dependent increase in Ca\textsuperscript{2+} peak amplitude (dfmr1\textsuperscript{vehicle}, 0.62 ± 0.09, n = 21 neurons; dfmr1\textsuperscript{ATR}, 0.57 ± 0.08, n = 15 neurons, t-test, p = 0.098; Fig. 6C) or decay half-life (dfmr1\textsuperscript{vehicle} t\textsubscript{1/2}, 6.27 ± 0.37 s, r\textsuperscript{2} = 0.94 ± 0.01, n = 21; dfmr1\textsuperscript{ATR} t\textsubscript{1/2}, 6.64 ± 0.56 s, r\textsuperscript{2} = 0.97 ± 0.01, n = 15, t-test, p = 0.57; Fig. 6C). However, there is an increase in time to peak (dfmr1\textsuperscript{vehicle}, 5.20 ± 0.51 s, n = 21; dfmr1\textsuperscript{ATR} t\textsubscript{1/2}, 10.02 ± 1.26 s, n = 15, t-test, p = 0.00036; Fig. 6C), an effect unique to this experimental paradigm. Thus, activity-dependent changes in Ca\textsuperscript{2+} signaling are again absent in dfmr1 null neurons, although we cannot rule out a stimulation-induced effect on broader timescale Ca\textsuperscript{2+} transients in
dfmr1 null MBON-11 neurons. Taken together, critical period stimulation of both neuron classes increases Ca\(^{2+}\) signals following depolarization, and this developmental change is completely dependent on FMRP.

4. Discussion

4.1. Neuron type-specific FMRP critical period requirements

The question of whether Fragile X syndrome (FXS) is a neurodevelopmental disease, a disease of continuous neural plasticity dysfunction, or some combination, is a question of paramount importance in designing effective disease interventions. The determination requires precise methods to dissect temporal requirements within defined neural circuitry. This study employs precisely-targeted transgenic drivers (Jenett et al., 2012) to introduce a [Ca\(^{2+}\)] reporter (GCamp5G) (Akerboom et al., 2012; Kirkhart and Scott, 2015) and optogenetic channelrhodopsin (ChR2) (Dani et al., 2014) into individually-identified excitatory input and inhibitory output neurons in the well-mapped MB learning/memory circuit (Tanaka et al., 2012; Tanaka et al., 2008). We previously discovered an early-use MB critical period defined by peak FMRP expression, in which FMRP loss prevents detection of sensory activity that shapes synaptic connectivity (Tessier and Broadie, 2008). More recently, we discovered excitatory input (mPN2) and inhibitory output (MBON-11) neurons display activity-dependent bidirectional responses to depolarizing and hyperpolarizing optogenetic manipulation, only within the FMRP-defined critical period and wholly dependent on FMRP, shaping synaptic connectivity (Doll and Broadie, 2015). We show here that FMRP bidirectionally regulates critical period calcium signaling in these two neuron classes: excitatory input neurons exhibit elevated depolarization-induced transients and inhibitory output neurons display reduced transients in dfmr1 mutants. These results reveal a novel critical period-specific mechanism supporting the E:I imbalance hypothesis of FXS (Gatto et al., 2014). It is well established that neural circuit optimization occurs via activity-dependent changes during restricted early-use temporal windows (Hensch, 2004; Holtmaat and Svoboda, 2009). A rich history of altered neural architecture in FXS (Irwin et al., 2001; Zhang et al., 2001), and recent functional dissections demonstrate that structural defects
are coupled with changes in neural excitability (Brager and Johnston, 2014). Importantly, mouse FXS models have also revealed essential roles for FMRP during critical period functional development. These include a pronounced developmental delay in excitatory somatosensory cortex (Harlow et al., 2010; Till et al., 2012), delayed depolarization-to-hyperpolarization switch in GABAergic transmission (He et al., 2014), and critical period-specific neural circuit hyperexcitation (Goncalves et al., 2013; Zhang et al., 2014). These pioneering studies of critical period refinement have provided the baseline for understanding the impact of FMRP on critical period neural circuit functional refinement, particularly in establishing and maintaining the proper excitatory-inhibitory balance (Contractor et al., 2015; Meredith, 2015).

In the Drosophila MB circuit, FMRP suppresses calcium signaling in excitatory input mPN2 neurons, but enhances calcium signaling in inhibitory output MBON-11 neurons (compare Figs. 2 and 3). These opposing roles for FMRP occur in starkly contrasting neuron classes: excitatory cholinergic projection neurons carry olfactory sensory input (Iniguez et al., 2013), and inhibitory GABAergic MBON-11 neurons (Aso et al., 2014a) maintain aversive memory for both olfactory and visual modalities (Aso et al., 2014b). Importantly, FXS has been characterized as a disease of E:I imbalance, with increased excitation and decreased inhibition (Gibson et al., 2008). This study provides additional evidence supporting this theory, with opposite calcium signaling defects in individually-identified excitatory vs. inhibitory neurons within the same learning/memory circuit. Although we cannot rule out a maintained role for FMRP at maturity, the defects in both neuron classes are far more pronounced during the critical period of initial sensory input. Most strikingly, both dfmr1 null neuron classes fail to undergo the dramatic developmental changes in calcium signaling that occur in wildtype animals. Conditional dfmr1 manipulations (cell autonomous rescue and RNAi) confirm the critical period specific FMRP requirement. However, we were surprised to find more robust dfmr1 knockdown effects within this transient period, compared to constitutive dfmr1 knockdown in both neuron classes. This may suggest that constitutive knockdown generates nonspecific effects, such as compensation due to RNAi expression in these neurons throughout development. Conditional critical period dfmr1 rescue provides a nearly complete restoration of calcium signaling in excitatory mPN2 neurons, but only a partial remediation of dfmr1 defects in inhibitory MBON-11 neurons. This may suggest that inhibitory neurons require a broader period of FMRP function during development, which may not be surprising given the persistent reduction in inhibitory signaling in this FXS disease model (Gatto et al., 2014).

4.2. FMRP control of calcium signaling during the critical period

How and why might FMRP mediate calcium signaling downstream of activity during critical period development? As an essential component of neuronal excitability, calcium influx and subsequent regulation represents a prime target for FMRP function (Tessier and Brodie, 2012). We postulate that impaired critical period calcium regulation may underlie formation of the inappropriate neural architecture and synaptic connectivity characterizing the FXS disease state (Lohmann, 2009; Tessier and Brodie, 2008; Tessier and Brodie, 2011). These functional defects may be rooted in defects in calcium buffering capacity caused by the loss of calcium-binding proteins (e.g. calmodulin and calbindin) in dfmr1 null mutants (Tessier and Brodie, 2011). Consistently, mutations in calmodulin and calbindin lead to altered calcium transients in multiple neuronal contexts (Arredondo et al., 1998; Barski et al., 2003). It is also possible that FMRP differentially regulates calcium release from intracellular stores in a neuron type-specific manner (Tessier and Brodie, 2011). Depolarization-dependent calcium influx can occur from the outside, from calcium store organelles within the neuron, or from a combination of both, and we have previously established that FMRP is involved in calcium mobilization from both pathways in Drosophila brain MB neurons (Tessier and Brodie, 2011). Importantly, calcium can regulate the structural (Lohmann et al., 2005; Lohmann and Wong, 2005) and functional (Lisman et al., 2002) adaptations necessary for synaptic specificity, and defective calcium signaling may underlie multiple autism phenotypes (Krey and Dolmetsch, 2007).

The direct binding and regulation of voltage-gated Ca\(^{2+}\) and K\(^+\) channels by FMRP represents an intriguing mechanism for the developmental maturation of circuits. A proper developmental analysis would need to include a temporal survey of channel expression in the FXS disease state, as well as roles for direct binding of FMRP to multiple classes of voltage-gated channels. For example, FMRP is capable of regulating both expression and degradation of Ca\(^{2+}\) channels (Ferron et al., 2014), thereby providing both direct and indirect regulatory roles. Interestingly, Ca\(^{2+}\) channels and sensors undergo strong developmental shifts in expression and spatial organization, for example in the calyx of Held (Fedchyshyn and Wang, 2005), including developmental alterations in the type of channels expressed following initial sensory onset (Alamilla and Gillespie, 2013). Likewise, the density of presynaptic K\(^+\) channels increases during late brain development, which correlates with shorter action potential duration (Nakamura and Takahashi, 2007). FMRP has been shown to maintain activity-dependent tonotopic K\(_{\text{r},3.1}\) expression gradients, with FMRP loss causing flattened tonotopicity and reduced K\(^+\) currents (Strumbos et al., 2010). Importantly, FMRP directly binds Na\(^{-}\)-gated KCNT1 and Ca\(^{2+}\)-gated BK\(^{\text{fi}}\) channel classes, which serve to repolarize the membrane following activity (Bean, 2007; Brown et al., 2010; Deng et al., 2013; Kim and Kaczmarek, 2014). The use of targeted dfmr1 mutations to disrupt RNA-binding translational regulation and channel-binding FMRP properties may allow us to begin to dissect these potential developmental mechanisms. For example, the newly described dfmr1 R138Q mutation disrupts BK channel binding without affecting translational repressive roles (Myrick et al., 2015). With such an array of regulatory capacities, FMRP modulation of channel expression and function will need to be explored both in the context of activity initiation through voltage-gated Ca\(^{2+}\) channels and activity termination via voltage-gated K\(^+\) channels, both of which could contribute to calcium signaling defects in the FXS disease state.

4.3. Optogenetic manipulations support FXS hyperexcitation theory

Combining optogenetic manipulation with precisely targeted neuron-specific transgenic drivers provides exciting new opportunities in developmental neuroscience (Honjo et al., 2012; Klapoetke et al., 2014). Use of these tools in the well-mapped Drosophila MB circuit is a particularly potent method to study activity-dependent neural development, especially to dissect cell autonomous requirements. Wildtype neurons respond to optogenetic stimulation during critical period development by strongly altering calcium signaling dynamics, but activity-dependent modulation is completely absent in dfmr1 null neurons. Single cell-targeted stimulation during the critical period leads to increased depolarization-induced calcium signaling, providing evidence of an activity-dependent alteration in functional properties and an exciting proof-of-principle of the hyperexcitation theory of FXS (Bear et al., 2004; Goncalves et al., 2013). Of particular interest, critical period stimulation of wildtype excitatory mPN2 neurons generates depolarization-dependent calcium dynamics strikingly reminiscent of dfmr1 null neurons, providing a specific illustration of the hyperexcitation theory of FXS (Gibson et al., 2008; Goncalves et al., 2013; Zhang et al., 2014). As projection neurons receive excitatory input from odorant receptor neurons (ORNs) in the antennal lobe (Vosshall and Stocker, 2007), this optogenetic hyper-excitation mimics exaggerated olfactory sensory input during circuit development. Thus, neurons are mis-tuned to inappropriate sensory input during the plasticity permissive critical period, resulting in calcium signaling impairments that closely mimic the FXS disease state.
Inhibitory GABAergic MBON-11 neurons also respond to heightened critical period stimulation with increased calcium signaling, demonstrating a similar activity-dependent response across neuron classes. However, this phenotype is specific to activity-manipulated wildtype neurons and does not phenocopy the dfmr1 null condition. This suggests that inhibitory MBON-11 neurons may not be exposed to developmental hyperexcitability in the FXS condition, as predicted for mPN2. MBON-11 neurons receive input from MB Kenyon Cell neurons (Aso et al., 2014a), but the neurotransmission mechanism remains elusive (Henry et al., 2012), despite an established essential role in aversive memory formation (Aso et al., 2014b). Since FMRP regulates calcium dynamics in an opposite direction in this inhibitory neuron type, the hyperexcitatory theory of FXS likely does not fully encompass the disease condition, which also includes hypo-inhibition as a prominent component. FMRP is known to regulate GABAergic components, in both Drosophila and mammals (Gatto et al., 2014; Lozano et al., 2014), and this likely provides a distinct FMRP regulatory mechanism on either end of the emerging E/I balance in the developing brain (Cea-Del Rio and Huntsman, 2014; Gibson et al., 2008). Unfortunately, the lack of activity response in dfmr1 null neurons may limit our ability to use exogenous activity modulation to dissect morphology and function. However, dfmr1 mutant neurons may display shifted critical periods (Harlow et al., 2010), and both the studied neuron classes possess calcium signaling dynamics that are much more similar to wildtype at maturity. With the application of more advanced optogenetics techniques, such as CsChrimson (Klapoetke et al., 2014), ChR2-XXL (Dawydow et al., 2014) and red-shifted chloride inhibitors like Jaws (Chuong et al., 2014), we may be able to more effectively dissect structural and functional components of developing and mature circuits.

5. Conclusions

This study contributes key new insights to our understanding of the FMRP requirements in activity-dependent critical period neural circuit refinement. The powerful Drosophila genetic toolkit has allowed us for the first time to define both neuron-specific and temporal-specific FMRP requirements in depolarization calcium signaling dynamics. The key results are 1) excitatory and inhibitory neurons are misregulated in opposite directions in the absence of FMRP, and 2) there is a restricted FMRP requirement during the early-use critical period. In the FXS disease state, excitatory (E) neurons exhibit elevated activity-dependent calcium transients and inhibitory (I) output neurons display suppressed transients, supporting the E:I imbalance hypothesis of FXS. Moreover, restricted critical period stimulation increases calcium transients in wildtype, and this developmental refinement depends absolutely on FMRP. Single cell-targeted optogenetic stimulation and FMRP conditional manipulations establish cell-autonomous critical period functional refinement mechanisms within individually identified single excitatory and inhibitory neurons within a common learning/memory circuit. Future use of targeted human patient FMRP point mutations will allow us to dissect the roles of RNA-binding translational regulation and channel-binding activity regulation in the control of these critical period developmental mechanisms.

Author contributions

C.A.D. and K.B. conceived and designed the experiments. C.A.D. performed all experiments and analyzed all data. C.A.D. and K.B. co-wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

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References


