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Drosophila fragile X mental retardation protein developmentally regulates activity-dependent axon pruning

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Fragile X Syndrome (FraX) is a broad-spectrum neurological disorder with symptoms ranging from hyperexcitability to mental retardation and autism. Loss of the *fragile X mental retardation 1* (*fmr1*) gene product, the mRNA-binding translational regulator FMRP, causes structural over-elaboration of dendritic and axonal processes, as well as functional alterations in synaptic plasticity at maturity. It is unclear, however, whether FraX is primarily a disease of development, a disease of plasticity or both: a distinction that is vital for engineering intervention strategies. To address this crucial issue, we have used the *Drosophila* FraX model to investigate the developmental function of *Drosophila* FMRP (dFMRP). dFMRP expression and regulation of *chickadee/profilin* coincides with a transient window of late brain development. During this time, dFMRP is positively regulated by sensory input activity, and is required to limit axon growth and for efficient activity-dependent pruning of axon branches in the Mushroom Body learning/memory center. These results demonstrate that dFMRP has a primary role in activity-dependent neural circuit refinement during late brain development.

KEY WORDS: Fragile X Syndrome, Mental retardation, Autism, Neural development, Translation, Synaptogenesis, Synaptic pruning

INTRODUCTION

Fragile X Syndrome (FraX) is a commonly inherited mental retardation disorder causing hyperactivity, hypersensitivity to sensory stimuli, epileptic seizures and autism (Belmonte and Bourgeron, 2006; Boccia and Roberts, 2000; Freund and Reiss, 1991; Sabaratnam et al., 2001). The fragile X mental retardation protein (FMRP) is an mRNA-binding protein proposed to function in mRNA trafficking, stability and translational regulation (Ashley, Jr et al., 1993; De Diego Otero et al., 2002; Feng et al., 1997; Laggerbauer et al., 2001; Weiler et al., 1997; Weiler et al., 2004; Xu et al., 2004; Zalfa et al., 2007; Zalfa et al., 2003; Zhang et al., 2007). Although FMRP potentially binds 4% of brain mRNAs, only a handful of targets have been validated (Brown et al., 2001; Hayashi et al., 2007; Zhang and Broadie, 2005). Accounting for this discrepancy may be that FMRP binds mRNA targets in response to neuronal activation (Bear et al., 2004). Neural activity from sensory experience and metabotropic glutamate receptor signaling increases FMRP expression and function (Gabel et al., 2004; Hou et al., 2006; Irwin et al., 2005; Irwin et al., 2000; Restivo et al., 2005; Todd and Mack, 2000; Todd et al., 2003; Valentine et al., 2000; Weiler et al., 1997). FMRP may repress translation via association with polyribosomes (Aschrafi et al., 2005; Khandjian et al., 2004; Stefani et al., 2004), transported to sites of local synaptic translation in response to neurotransmission (Ferrari et al., 2007; Greenough et al., 1985; Ostroff et al., 2002).

Activity plays crucial roles in sculpting neural circuits during development and later in mediating plasticity (Desai et al., 2002; Zito and Svoboda, 2002). FMRP may perform a common function in regulating activity-dependent protein synthesis in both settings. In FraX patients, mutant mice and *Drosophila*, dendritic arbors are overgrown with immature dendritic spines, suggesting a failure of synapse maturation (Comery et al., 1997; Galvez et al., 2003; Galvez and Greenough, 2005; Galvez et al., 2005; Grossman et al., 2006; Irwin et al., 2002; Irwin et al., 2001; Ivanco and Greenough, 2002; McKinney et al., 2005; Nimchinsky et al., 2001; Pan et al., 2004; Rudelli et al., 1985). Dendritic defects are robust during early postnatal development and abrogate with maturation (Galvez and Greenough, 2005; Nimchinsky et al., 2001). Similarly, mutant neurons exhibit axonal over-branching in mice and *Drosophila*, indicating a similar presynaptic requirement (Antar et al., 2006; Pan et al., 2004). FMRP is also required for plasticity in mature synapses; long-term depression (LTD) is enhanced (Hou et al., 2006; Huber et al., 2002; Koekkoek et al., 2005) and long-term potentiation (LTP) is reduced (Larson et al., 2005; Li et al., 2002; Zhao et al., 2005). These data suggest two roles for FMRP: during development to regulate the structuring of neural circuits and during maturity to regulate maintained plasticity.

Drosophila is well suited for the dissection of developmental processes; however, the well-characterized *Drosophila* FraX model has yet to be exploited for this purpose. Therefore, we investigated the developmental roles of *Drosophila* FMRP (dFMRP) in the *Drosophila* brain, specifically the activity-dependent structural changes driven by sensory input. We found that dFMRP expression and function are maximal during late-stage periods of axon pruning, which requires both dFMRP and sensory input activity. These results reveal a prominent role for dFMRP in activity-dependent neural circuit refinement.

MATERIALS AND METHODS

Fly stocks and genetics

The genetic wild-type strain was w^{1118}. The *Drosophila fmr1* (*dfmr1*)-null allele was *w*; $dfmr1^{50M}$/*TM6GFP*. The control for MARCM analyses was obtained by crossing *heatshock-FLP, mCD8-GFP; FRT82B,Tubulin P-Gal80/Sb; Gal4-OK107* with *y, w; FRT82B/Sb*. The control for sensory stimulation experiments was crossed to *y, w; FRT82B,Sb/TM6*. The mutant MARCM chromosome was *FRT82B,*$dfmr1^{50M}$*/TM6GFP* and the overexpression chromosome was *FRT82B,*$dfmr1^{50M}$*,UAS-dfmr1/TM6GFP*. Standard techniques produced two recombinant channelrhodopsin-2 lines: *FRT82b,UAS-CHR2/TM6* and *FRT82b,*$dfmr1^{50M}$*,UAS-CHR2/TM6GFP*.

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Sensory mutants used were *Or83b-2* (provided by Dr Leslie Vosshall, Rockefeller University, New York, NY) and *ninaE* (provided by Dr Bih-Hwa Shieh, Vanderbilt University, Nashville, TN).

Protein and RNA extraction

Fly heads were frozen on dry ice and stored at –80°C. Protein/RNA was extracted from the same samples of 10-25 pooled heads using Trizol (Invitrogen, Carlsbad, CA). Protein pellets were resuspended in 8 M Urea, 1% SDS supplemented with 1×Complete Protease Inhibitors (Roche, Indianapolis, IN) incubated at 50°C for 1 hour with intermittent vortexing. Protein concentration was determined using a MicroBCA Assay (Pierce, Rockford, IL). RNA pellets were resuspended in DEPC-treated water, and concentration determined by absorbance at 260 nm.

Western blotting

Single heads were homogenized in 1×Nupage LDS Sample Buffer (Invitrogen, Carlsbad, CA) with 55 mM DTT. Debris was pelleted by centrifugation at 16,000× ***g*** at 25°C and samples boiled for 10 minutes. Extracts were loaded onto a 4-12% Bis-Tris gel and electrophoresed at 200 V in 1×MOPS or 1×MES buffer (Invitrogen, Carlsbad, CA). Protein was transferred to nitrocellulose in 1×Nupage Transfer Buffer (Invitrogen, Carlsbad, CA) plus 10% methanol at 100 V for 1 hour. The membrane was blocked for 1 hour in Odyssey Blocking Buffer (Li-Cor, Lincoln, NE) and probed for 12-16 hours at 4°C with the following antibodies: dFMRP, 6A15 (Sigma, St Louis, MO), 1:5000 (for developmental blots) or 1:500 (for sensory-deprivation blots); Chickadee/Profilin, Chi1J (Developmental Studies Hybridoma Bank, Iowa City, IA), 1:10; α-Tubulin (Sigma, St Louis, MO), 1:400,000. Membranes were washed three times with buffer (25 mM Tris pH 8.0, 150 mM sodium chloride, 0.05% Ige-PAL-CA630). The secondary antibody, anti-mouse IgG IR800 (Rockland, Gilbertsville, PA) was diluted 1:10,000 in Odyssey Blocking Buffer and applied for 1 hour at 25°C. The blot was washed three times with buffer and then scanned on the Odyssey Infrared Imaging System.

Quantitative RT-PCR

cDNA was made from DNase I Turbo-treated (Ambion, Austin, TX) RNA (2 μg) using random hexamer primers and Superscript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR of cDNA (1 μl) was carried out using SYBR Green JumpStart Taq Ready Mix (Sigma, St Louis, MO). The following primers were used at 0.5 μM concentrations each per reaction:

gapdh2, 5′-CCTTGCAAGCAAGCCGATAG-3′, 5′-CGACATGGTTAACTTTTTGT-3′;

dfmr1, 5′-GTTCGGCTCGACAATGGCGC-3′, 5′-GCGACAGCTGTCACCTGGCC-3′;

chickadee, 5′-CGCAGTCCAGTGGCTTTGAG-3′, 5′CGCTGATCAGTTTGGAGAGC-3′.

Cycling parameters were 95°C for 3 minutes, then 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds for 40 cycles (Bio-Rad iQ5 Thermal Cycler). Each experiment consisted of three biological replicates for each time point plated in duplicate.

Immunocytochemistry

Brains were dissected in 1×PBS and fixed in 4% paraformaldehyde + 4% sucrose in 1×PBS for 40 minutes at 25°C. Brains were washed three times with buffer (1×PBS, 1% BSA, 0.1% Triton X-100) for 30 minutes and incubated with the following primary antibodies at 4°C for 12-16 hours: dFMRP, 6A15 (Sigma, St Louis, MO), 1:250; mouse CD8 (Caltag, Burlingame, CA), 1:50; FasII (Developmental Studies Hybridoma Bank 1D4, Iowa City, IA), 1:10. Secondary antibodies, anti-mouse-IgG-Cy3 and anti-rat-IgG-Cy5 (Jackson ImmunoResearch, West Grove, PA), and anti-mouse-IgG AlexaFluor 488 (Molecular Probes, Eugene, OR), diluted 1:400 were applied for 2-3 hours at 25°C. Brains were washed three times for 1 hour before mounting in Vectashield (Vector Labs, Burlingame, CA) and imaging on a Zeiss Meta 510 confocal microscope. Images were collected at identical settings and presented as maximum *z*-projections. As previously (Pan et al., 2004), MARCM branch parameters were determined with LSM software on 3D confocal *z*-stacks of each neuron.

RESULTS

dFMRP developmentally regulates brain RNA and protein levels

FMRP negatively regulates protein translation in the mature brain, but little is known about FMRP developmental requirements (Khandjian et al., 2004; Laggerbauer et al., 2001; Li et al., 2001; Qin et al., 2005; Sung et al., 2003). Research on *Drosophila* brain development has focused on the first 48 hours during pupal metamorphosis (Awasaki and Ito, 2004; Awasaki et al., 2006; Brown et al., 2006; Lee et al., 1999; Marin et al., 2005; Watts et al., 2004; Williams and Truman, 2005; Zheng et al., 2003), but less is known about the latter half of brain development. However, presumed roles for activity and FMRP coincide with this time of use-dependent process refinement (Boothe et al., 1979; Desai et al., 2002; Fox and Wong, 2005; Hinds and Hinds, 1976; Huttenlocher, 1979; Lund et al., 1977; Pan et al., 2004; Stern et al., 2001; Turrigiano and Nelson, 2004). To assay the temporal requirements for dFMRP during brain development versus maturity, we analyzed developmental time points beginning 60 hours after puparium formation (APF) and extending into the mature adult (9 days post-eclosion).

Null *dfmr1* mutants (*dfmr1*^{50M}) were compared with controls (w^{1118}) at mid-pupal day 3 (P3; 60-72 hours APF), mid-pupal day 4 (P4; 88-96 hours APF), immediately post-eclosion [0-7 hours after eclosion (AE)], and at 1 day (1d), 4 days (4d) and 9 days (9d) in the adult. Total RNA was quantified as μg per head (Fig. 1A). The total amount of RNA was higher in both wild type (WT) and mutants during stages of pupal brain development than at maturity. In *dfmr1* nulls, there was a significant increase in RNA only during a restricted window of late pupal development (Fig. 1A). There was a 38% (P3) and 51% (P4) increase in total RNA in the *dfmr1* nulls compared with wild type (P3: WT, 0.58±0.14 μg, *dfmr1*, 0.79±0.08 μg, P=0.025, n=6; P4: WT, 0.47±0.17 μg, *dfmr1*, 0.71±0.11 μg, P=0.016, n=6). Conversely, there were no significant differences in RNA levels throughout all adult time points (Fig. 1A). Thus, dFMRP functions to negatively regulate RNA levels during a restricted window of late pupal brain development.

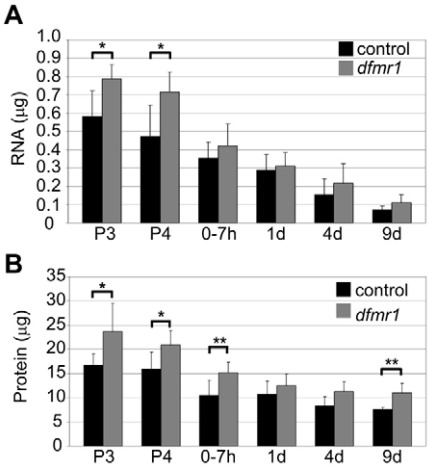

Fig. 1. dFMRP regulates protein and RNA levels during brain maturation. Total RNA (**A**) and protein (**B**) at indicated developmental time points from *dfmr1* nulls (*dfmr1*^{50M}) and controls (*w*^{1118}). Bars show the mean μg per head ± standard deviation. *0.05>P>0.01; **0.01<P<0.001. Stages: P3, 60-70 hours APF; P4, 88-96 hours APF; 0-7h, 0-7 hours AE; 1d, 21-24 hours AE; 4d, 96-112 hours AE; 9d, 216-232 hours AE.

As with total RNA, total protein from wild type and mutants was higher in the developing brain than in the mature brain. There was a 2-fold decrease in protein (μg/head) over the 11-day assay period (Fig. 1B). The *dfmr1* nulls had significantly elevated protein during a restricted window of development, with elevated protein levels persisting into the early-use period following eclosion (Fig. 1B). There were significant protein increases of 42% (P3), 31% (P4) and 44% (0-7 hours AE) in *dfmr1* nulls compared with wild type (P3: WT, 16.7±2.5 μg, *dfmr1*, 23.73±5.8 μg, P=0.01, n=6; P4: WT, 16.0±3.4 μg, *dfmr1*, 20.9±3.0 μg, P=0.04, n=6; 0-7 hours: WT, 10.5±3.1 μg, *dfmr1*, 15.2±2.1 μg, P=0.001, n=12). There were no significant differences in adult animals for several days following this early requirement (Fig. 1B). However, a new period of requirement in the mature brain occurred at nine days, when the *dfmr1* null contained 43% more protein than did wild type (9d: WT, 7.68±0.4 μg, *dfmr1*, 11.0±2.0 μg, P=0.004, n=6). These results suggest that there is a transient window of dFMRP requirement during late pupal development extending into the early-use period of the young adult, followed by a separable requirement much later in the mature brain.

dFMRP expression and function is developmentally regulated

The restricted window of RNA/protein upregulation during late *dfmr1* brain development/early adult use, suggested that dFMRP expression itself might be similarly developmentally regulated. FMRP is expressed at higher levels during development in mammals (Khandjian et al., 1995; Lu et al., 2004; Singh et al., 2007; Wang et al., 2004), but there is no indication of whether developmental regulation occurs at the level of transcription or translation. FMRP represses its own translation (Ashley, Jr et al., 1993; Brown et al., 1998; Ceman et al., 1999; Schaeffer et al., 2001; Sung et al., 2000), adding a complicating factor to understanding the mechanism of regulation. To address this issue, *dfmr1* mRNA and dFMRP protein were assayed from pupal development to maturity in wild-type animals.

dfmr1 mRNA levels were measured by quantitative RT-PCR during the same time points as above, but narrowing the eclosion time point to 0-3 hours AE and including 7 days AE to better define the late expression profile. mRNA levels were normalized to a housekeeping gene, GAPDH2, and reported as a fold change relative to the first time point (Fig. 2A). The expression of *dfmr1* mRNA follows two distinct patterns. First, there is high abundance during pupal brain development, which then falls rapidly upon eclosion and remains low for the first day of adult life (Fig. 2A). This pattern is consistent with the reduction in overall RNA abundance during the development to adult transition, as shown in Fig. 1A. The abundance of *dfmr1* mRNA, however, increases 50% between days 1 and 4 (P=0.009). In the adult (>7 days post-eclosion), *dfmr1* message remains elevated to levels similar to those at pupation (Fig. 2A). This second period of *dfmr1* mRNA elevation is divergent from the total RNA profile, which declines into maturity (Fig. 1A). Thus, there are two distinct phases of *dfmr1* transcription, with one peak during late brain maturation and a second comparable plateau in the fully mature brain.

The dFMRP protein level in the brain was measured by both immunoblot and immunocytochemistry. Western blot analyses were performed on heads from single animals, in duplicate, from seven developmental time points (Fig. 2B). As predicted from the *dfmr1* mRNA levels, dFMRP protein expression is maximal during late stages of brain development and decreases during the first day post-eclosion (Fig. 2B). At maturity (>1 day), dFMRP protein was difficult to detect on western blots and, surprisingly, no longer

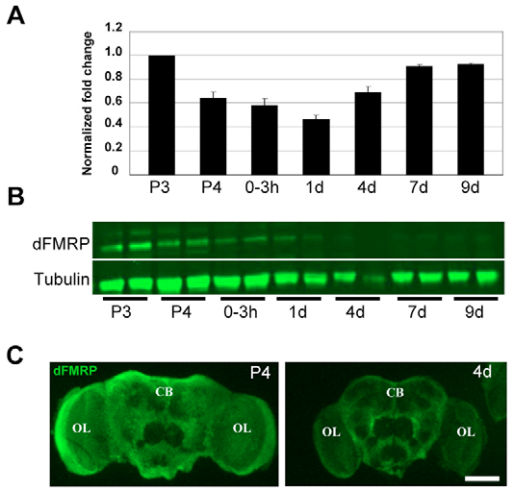

Fig. 2. dFMRP protein and mRNA are differentially developmentally regulated. (**A**) Quantitative RT-PCR of *dfmr1* mRNA levels normalized to GAPDH2 and reported as fold changes relative to the first time point. Bars show mean±s.e.m. (**B**) Western blot analysis of dFMRP protein. Each lane represents a single head at indicated stages (2 heads per stage). α-Tubulin is the loading control. (**C**) Immunocytochemistry of dFMRP in control (w^{1118}) brains. CB, central brain; OL, optic lobe. Scale bar: 100 μm. Stages: P3, 60-70 hours APF; P4, 88-96 hours APF; 0-3h, 0-3 hours AE; 1d, 21-24 hours AE; 4d, 96-112 hours AE; 7d, 168-184 hours AE; 9d, 216-232 hours AE.

correlated with *dfmr1* mRNA levels (compare Fig. 2A with 2B). Although *dfmr1* mRNA was high 4 days AE, dFMRP protein levels remained minimal. This expression profile was also evident by brain immunocytochemistry (Fig. 2C). During late pupation, dFMRP is expressed at high levels throughout the entire brain, primarily in neuronal soma, whereas in the mature brain dFMRP expression is strongly reduced, except in limited central brain regions. These data show that *dfmr1* mRNA and dFMRP protein levels correlate closely during brain development, but that *dfmr1* transcription and translation become uncoupled in the mature brain.

To test whether dFMRP function is similarly regulated in the same developmental pattern, we examined the dFMRP target *chickadee/profilin* (Reeve et al., 2005). Actin-binding Chickadee is upregulated in the absence of dFMRP, but nothing is known about its developmental regulation. We performed quantitative RT-PCR for *chickadee* mRNA on *dfmr1*-null and wild-type extracts from development through maturity (Fig. 3A). In wild type, the amount of *chickadee* mRNA nearly doubles during late pupation and then falls precipitously after eclosion, and remains low in the adult brain (Fig. 3A). In *dfmr1* mutants, *chickadee* mRNA levels follow a similar profile with two important differences. First, the P4 spike is approximately three times the level of mRNA at P3 and is 46% increased over controls. Second, by 9 days AE, *chickadee* mRNA levels are actually decreased (43%) compared with controls (Fig. 3A). Wild-type protein levels are maximal during pupal day 4 and rapidly fall at eclosion (Fig. 3B), correlating with the mRNA levels. By contrast, Chickadee protein in *dfmr1* nulls is maintained at aberrantly high levels following eclosion and through the early-use period in the young adult (Fig. 3B). No protein changes are apparent at 9 days when *chickadee* mRNA levels are reduced in the mutant. These findings are consistent with distinct developmentally controlled roles for dFMRP function during late-brain development/early-use refinement periods and at maturity.

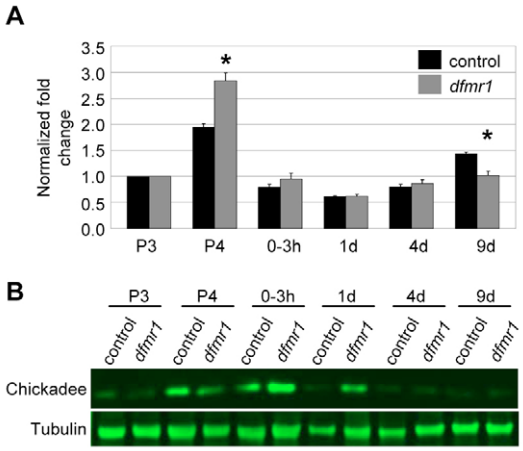

Fig. 3. The dFMRP target *chickadee/profilin* is developmentally regulated. (**A**) Quantitative RT-PCR of *chickadee/profilin* mRNA normalized to GAPDH2 and reported as fold changes relative to the first time point. Bars show mean±s.e.m. (**B**) Western blot analysis of Chickadee/Profilin from control and *dfmr1* animals (one head per lane) at the developmental time points shown. α-Tubulin is the loading control.

dFMRP regulates a late development period of axonal pruning

The Mushroom Body (MB) is a primary learning/memory center in the *Drosophila* brain, and is therefore the focus of behavioral, structural and functional studies (Margulies et al., 2005; Zars et al., 2000). *dfmr1*-null MB neurons exhibit increased axonal growth and over-branching (Pan et al., 2004). However, these analyses were carried out in unstaged brains and the cause of the defects was not determined. As dFMRP expression and function is differentially regulated during development and maturity, we re-examined MB neuron axon morphogenesis throughout development. The Mosaic Analysis with a Repressible Cell Marker (MARCM) genetic clonal technique (Lee and Luo, 2001) was used to label single homozygous mutant MB gamma neurons (Fig. 4A). This method allows for the direct visualization of individual neuron structure in the intact brain, and also permits direct analysis of the cell-autonomous function of dFMRP in that single neuron.

MARCM analyses were performed on single control and *dfmr1*-null MB neurons with MB axonal lobes defined with anti-Fasciclin II (FASII, red, Fig. 4A). The gamma neuron class was selected for analysis because of its well-defined morphology and simple single axon projection (Fig. 4A, green). Single neuron structure was analyzed at four time points: P3, P4, 0-3 hours AE and 4 days AE (Fig. 4B). Just prior to eclosion, both control and *dfmr1*-null neurons undergo a period of increased axonal growth as the total length of branches increases significantly (Fig. 4B). Although there were no differences in branch number or axon length at pupal day 3, by pupal day 4 *dfmr1*-null axons had grown 25% longer than control axons (P=0.02), although the overall branch number remained comparable (Fig. 4B). However, *dfmr1*-null neurons at P4 contained 31% more large branches (>10 μm) and 26% fewer small branches (<5 μm) than did control neurons (P=0.004 and P=0.026, respectively). Thus, dFMRP negatively regulates MB axon branch growth specifically during late stages of pupal brain development.

Eclosion heralds the onset of use-dependent activity in brain circuits. In control neurons at eclosion, there were decreases in both the overall branch length and the branch number relative to P4,

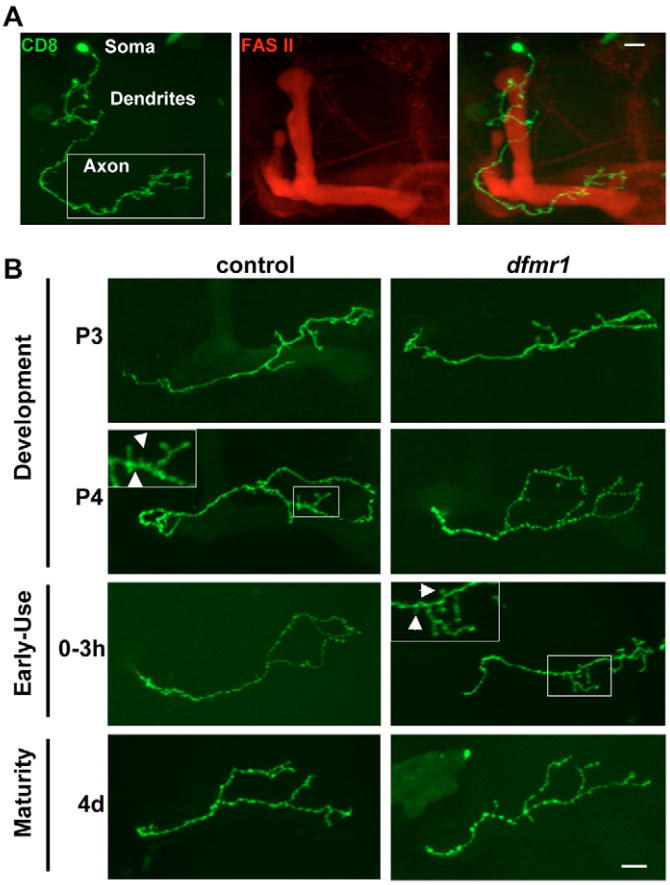

Fig. 4. Single-cell MARCM clonal analysis of MB gamma neuron development. (**A**) Representative image of Fasciclin II (FASII, red) labeled Mushroom Body containing a single MARCM gamma neuron clone (green). The white box highlights the area of axonal projection. (**B**) Developmental profile of axon projections of single-cell MARCM gamma neuron clones. Boxed insets are magnifications of areas of small (<5 μm) presynaptic branches (arrowheads), which are subject to pruning. P3, 60-70 hours APF; P4, 88-96 hours APF; 0-3h, 0-3 hours AE; 4d, 96-112 hours AE. Scale bar: 10 μm.

which is consistent with a pruning mechanism (Fig. 4B). The specific branches pruned were short processes (<5 μm; Fig. 4B, inset arrows). By contrast, *dfmr1*-null neurons had no significant decrease in axon branches during this normal pruning period. As a result, *dfmr1*-null neurons had 24% more and 30% longer axonal branches than controls immediately following eclosion (P=0.02 and P=0.003, respectively; Fig. 4B). In 4 day AE controls, when dFMRP protein is minimally expressed (Fig. 2B), both the branch number and length were static. Conversely, pruning that was absent in *dfmr1*-null neurons post-eclosion manifested by 4 days AE (Fig. 4B). Again, primarily branches <5 μm were pruned by this mechanism (Fig. 4B, inset). Interestingly, the excessive branching in *dfmr1*-null neurons present in young animals was absent in mature adults (number and length: P=0.17, 0.84, respectively). In fact, over-pruning is evidenced by a 35% decrease in small (<5 μm) axon branches in *dfmr1*-null neurons when compared with controls at maturity (P=0.01; Fig. 4B).

The overexpression of dFMRP causes under-branching of gamma neurons, the inverse of the *dfmr1*-null phenotype (Pan et al., 2004). Therefore, we examined the developmental profile of single-

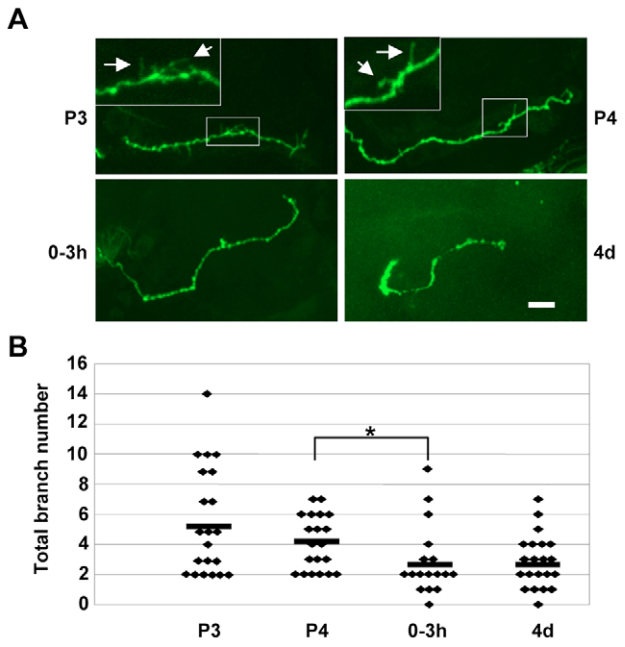

Fig. 5. dFMRP overexpression abrogates normal MB neuronal development. (**A**) Developmental profile of axon projections of single-cell MARCM gamma neuron clones overexpressing dFMRP. Boxed insets highlight presynaptic branches that are pruned (arrows). Scale bar: 10 μm. (**B**) Quantitation of total axon branch number of dFMRP-overexpressing MARCM clones. Each point is data from a single-cell MARCM clone. Horizontal lines represent the mean for each data set. *0.01<*P*<0.05. P3, 60-70 hours APF; P4, 88-96 hours APF; 0-3h, 0-3 hours AE; 4d, 96-112 hours AE.

cell MARCM clones overexpressing (OE) dFMRP. At P3, underbranching is already apparent with 47% fewer branches (*P*=0.002), which were on average 61% shorter (*P*<0.001) than controls (Fig. 5A). The axon growth that normally occurs during P4, fails in dFMRP OE neurons, but branch number and length remain unchanged [Fig. 5B; number: P3, 5.5±3.6 (*n*=21); P4, 4.1±1.8 (*n*=20); length: P3, 25.5±11.6 μm (*n*=21); P4, 20.5±8.7 μm (*n*=20)]. This undergrowth persists into maturity at 4 days post-eclosion and is worsened by aberrant excessive pruning immediately after eclosion. Axon branch number in OE neurons decreases by ~30% (*P*=0.03) upon eclosion [Fig. 5B; P4, 4.1±1.8 (*n*=20); 0-3 hours AE, 2.9±2.4 (*n*=17)]. The only branches available for pruning are the short filipodial-like branches persistent throughout late pupation (Fig. 5A, insets). Thus, dFMRP overexpression inhibits axonal elongation and accelerates post-eclosion process refinement.

Activity-dependent regulation of dFMRP expression and function

The pruning of small axonal branches in MB neurons occurs concomitantly with the onset of use, consistent with a period of refinement to remove weak or improperly formed synapes. The observation that this pruning mechanism is delayed beyond the early-use period in *dfmr1*-null neurons suggests that activity may regulate dFMRP function. To test this hypothesis, we performed sensory-deprivation experiments to block the external stimulation that should be required for the use-dependent testing of connectivity. Forty-eight hour old individual pupae were isolated in 1.5 ml tubes filled with 1 ml of food to prevent social stimulation and limit exploration/movement. Tubes were maintained in total darkness to prevent visual stimulation and kept in isolation boxes to mitigate auditory stimulation. Animals were allowed to develop under these conditions (sensory-deprived; SD) until 4 days AE. Controls developed in standard fly vials with ~30 other animals and were maintained in 12:12 light/dark conditions until 4 days AE.

FMRP expression has been reported to be elevated in response to sensory and neuronal stimulation (Gabel et al., 2004; Hou et al., 2006; Irwin et al., 2005; Irwin et al., 2000; Todd and Mack, 2000; Todd et al., 2003; Valentine et al., 2000; Weiler et al., 1997). Therefore, we first tested whether dFMRP expression was affected by sensory deprivation. The abundance of *dfmr1* mRNA was assayed by quantitative RT-PCR (Fig. 6A). The *dfmr1* mRNA level was reduced by ~20% in SD animals (*P*=0.02). Western analyses on single heads showed dFMRP protein levels were also significantly reduced (*P*=0.04; Fig. 6B), by ~20%, in SD animals (seven pairs of control and SD heads, three trials; Fig. 6C). Thus, sensory input activity positively regulates dFMRP mRNA and protein levels.

To refine these analyses to specific sensory modalities, we next assayed dFMRP expression in two sensory transduction mutants: *Or83b*, which eliminates a widely expressed odorant co-receptor required for olfaction of a broad spectrum of odors; and *ninaE*, which eliminates rhodopsin required for visual phototransduction (Larsson et al., 2004; O'Tousa et al., 1985). By single head western analysis, there was a comparable ~20% reduction in dFMRP expression in both mutants (Fig. 7A,B), demonstrating that vision and olfaction positively regulate dFMRP. Importantly, there was also a significant, opposing increase in the dFMRP target *chickadee*/*profilin* (Fig. 7A,B). Together, these results demonstrate that neural activity driven by sensory input is a positive regulator of dFMRP expression and function.

We next assayed the effect of sensory input activity on MB axon pruning (Fig. 8). Single MB gamma neuron MARCM clones were analyzed in animals grown until 4 days AE in normal versus SD conditions. SD animals displayed a significant 34% (*P*=0.03) increase in axon branch number in control neurons, but a highly significant 86% (*P*=0.001) increase in *dfmr1*-null neurons (Fig. 8A).

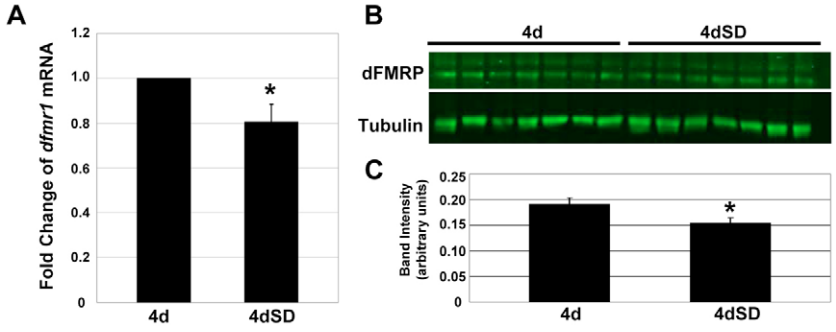

Fig. 6. Sensory input deprivation reduces dFMRP expression. (**A**) Quantitative RT-PCR of *dfmr1* mRNA normalized to GAPDH2 from normally reared and sensory-deprived (SD) animals at 4 days (96-112 hours AE). Bars show the mean±s.e.m. (*n*=4). *0.05>*P*>0.01. (**B**) Western blot for dFMRP of normally reared and SD animals at 4 days (96-112 hours AE), with one head per lane. (**C**) Quantitation of dFMRP western blot normalized to α-Tubulin. Bars show the mean±s.e.m. *0.05>*P*>0.01.

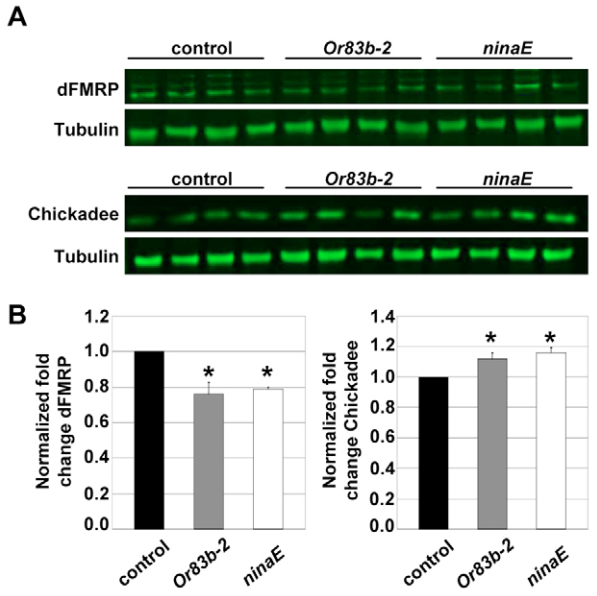

Fig. 7. Olfactory and vision mutants reduce dFMRP expression and function. (**A**) Western blot analysis of dFMRP and Chickadee/Profilin in control, odorant receptor mutant *Or83b*, and rhodopsin mutant *ninaE* animals. Single heads from <16-hour-old animals loaded per lane. α-Tubulin is the loading control. (**B**) Quantitation of dFMRP and Chickadee/Profilin western analysis. Bars show the mean±s.e.m. (n=6). *0.05>P>0.01.

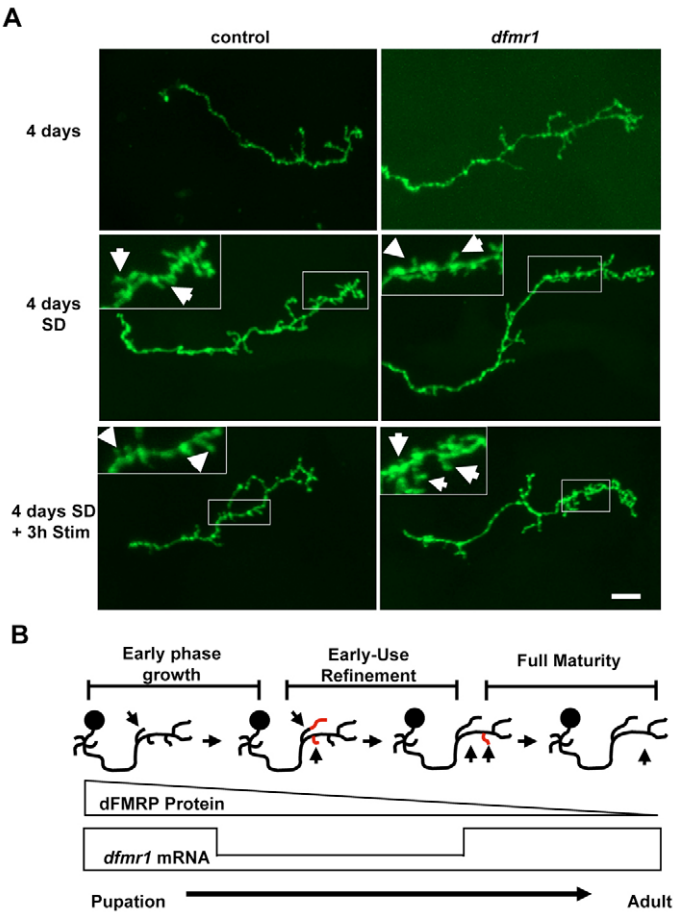

Fig. 8. Sensory-deprivation modifies dFMRP-dependent axon pruning. (**A**) Representative images of single-cell MARCM gamma neuron axon projections at 4 days post-eclosion, from animals raised in standard conditions (top row), sensory-deprived conditions (middle row) and sensory-deprived conditions followed by 3 hours of normal sensory stimulation (bottom row). Boxed insets highlight small (<5 μm) branches (arrows). Scale bar: 10 μm. (**B**) Diagram of dFMRP-dependent changes in MB axonal projections. dFMRP protein and mRNA are expressed maximally during late pupation and the early-use period after eclosion. Activity-dependent pruning in MB axons occurs during this window, dependent on dFMRP. At maturity, transcriptional and translational regulation of *dfmr1* becomes uncoupled, as mRNA levels inversely correlate with protein levels.

Axon branch length did not increase in control neurons, but was significantly (P<0.001) increased by 47% in *dfmr1*-null neurons. As a consequence, after 4 days of sensory deprivation, *dfmr1*-null axon branches were 30% longer than controls (P=0.002; Fig. 5A). The failure to prune was most apparent in *dfmr1*-null neurons in branches less than 5 μm in length (P<0.001), but was also apparent in longer branches binned between 5 and 10 μm (P<0.001).

Pruning normally occurs within 3 hours post-eclosion. As sensory deprivation inhibits pruning, we wanted to determine whether pruning could be restored with a subsequent activity period of the same duration. To test this possibility, 4-day SD animals were acutely stimulated with normal rearing conditions for 3 hours immediately prior to analysis. Neurons from these animals were identical to SD animals (Fig. 8A), with 24% longer *dfmr1*-null axon branches than controls (P=0.002). Thus, there is a restricted developmental window in which highly expressed dFMRP normally mediates use-dependent pruning of axonal branches, and, once this window has passed and dFMRP is no longer expressed at high levels, the normal rapid pruning mechanism cannot occur (Fig. 8B).

Neuronal activation enhances dFMRP-dependent axon pruning

Because sensory-deprivation blocks pruning, inducing neuronal activity may increase pruning. To test this hypothesis, we generated MARCM clones expressing *Chlamydomonas reinhardtii* light-gated channelrhodopsin-2 (CHR2) (Nagel et al., 2003). In the presence of the cofactor all-trans retinal, CHR2 channels open in response to blue light (Schroll et al., 2006). We generated recombinant MARCM CHR2 animals in the control or in the *dfmr1* null mutant. Flies were fed all-trans retinal or an ethanol vehicle throughout development. Upon eclosion (<12 hours AE), both genotypes were subjected to 1-Hz blue light (470 nm) pulses for 6 hours. Brains were dissected and single-cell gamma neuron MARCM clones analyzed (Fig. 9A).

Neuronal activation of MB neurons in CHR2-expressing clones resulted in a significant 21% reduction (P=0.02) in the total number of axonal branches in animals fed the retinal cofactor compared with vehicle-fed controls (Fig. 9B). Importantly, small axon branches (<5 μm) that were not pruned during sensory deprivation were reduced 27% by neuronal activation (Fig. 9C). Induced pruning was totally dependent on dFMRP, as no effect was observed in *dfmr1*-null neurons expressing CHR2 (Fig. 9). Thus, pruning of axon branches during the early use-refinement phase requires both neuronal electrical activity and dFMRP function.

Two-phase dFMRP requirement: axonogenesis and activity-dependent pruning

The quantification of neuronal architecture reveals two phases of dFMRP regulation in the MB: axon growth during late-stage pupation and activity-dependent axon pruning during the early-use

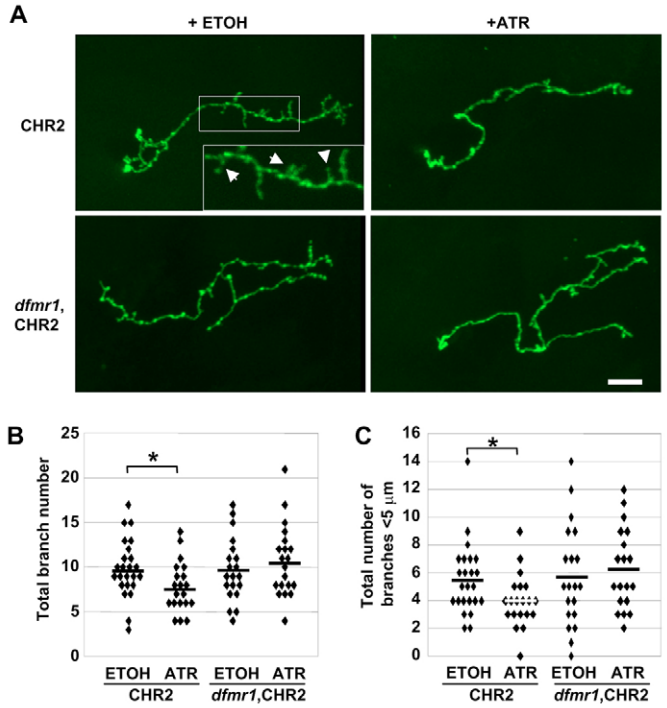

Fig. 9. CHR2-induced neuronal activation drives dFMRP-dependent pruning. (**A**) Single-cell MARCM clones expressing channelrhodopsin-2 (CHR2) in control or in *dfmr1*-null backgrounds, from animals grown on food containing all-trans retinal (ATR) or ethanol (ETOH vehicle). Post-eclosion animals (<12 hours AE) were stimulated with 470 nm light at 1 Hz pulses for 6 hours. Scale bar: 10 μm. (**B**,**C**) Quantified total axon branch number (B) and <5 μm branch number (C) of CHR2-expressing MARCM clones after stimulation. Horizontal bars are the mean for each data set. *0.01<*P*<0.05.

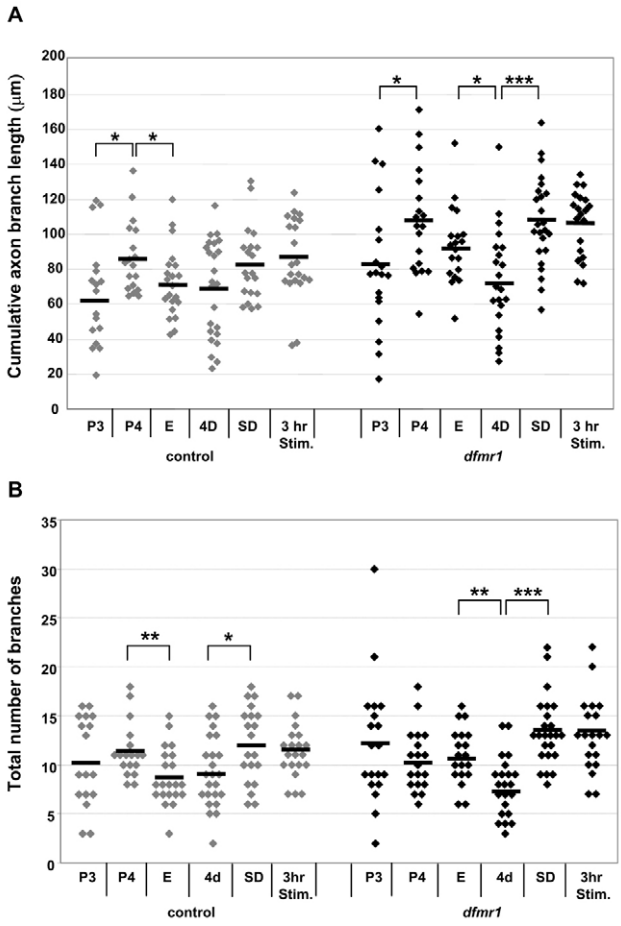

Fig. 10. Developmental changes in axon branch length and number in Mushroom Body clones in control and *dfmr1*-null mutants. Cumulative axon branch length (**A**) and total axon branch number (**B**) of gamma neurons from the indicated genotypes and stages. Each point is data from a single-cell MARCM clone. Horizontal lines represent the mean for each data set. P3, 60-70 hours APF; P4, 88-96 hours APF; E, 0-3 hours AE; 4D, 96-112 hours AE; SD, sensory-input deprived (96-112 hours AE); 3hr Stim, 3 hours sensory stimulation (sensory deprived for 4 days followed by 3 hours of sensory input stimulation). *0.01<*P*<0.05; **0.001<*P*<0.01; ***P*<0.001.

phase following eclosion. The cumulative length of axon branches was significantly increased in *dfmr1*-null neurons during P3/P4 [WT: P3, 66.1±30.2 μm (*n*=17), P4, 86.5±20.6 μm (*n*=18), *P*=0.03; *dfmr1*: P3, 82.8±38.3 μm (*n*=19), P4, 108.1±30.7 μm (*n*=19), *P*=0.02; Fig. 10A]. By contrast, the number of axon branches was not changed during this development period [WT: P3, 10.5±4.5 (*n*=17), P4, 11.4±2.8 (*n*=18), *P*=0.5; *dmfr1*: P3, 12.2±6.3 (*n*=19), P4, 10.5±3.2 (*n*=19), *P*=0.4; Fig. 10B]. Thus, dFMRP regulates axon growth but not branching during late-stage brain development.

At eclosion, MB neurons normally decrease in both axon branch length and number [length: 0-3 hours AE, 72.1±19.8 μm (*n*=21), *P*=0.02 relative to P4 (Fig. 10A); number: 0-3 hours AE, 8.8±2.9 (*n*=21), *P*=0.007 relative to P4; Fig. 10B]. By contrast, *dfmr1*-null neurons display no such pruning at eclosion [length: 0-3 hours AE, 93.7±21.9 μm (*n*=19), *P*=0.11 relative to P4 (Fig. 10A); number: 0-3 hours AE, 10.9±2.8 (*n*=19), *P*=0.48 relative to P4; Fig. 10B]. Instead, pruning is delayed in *dfmr1*-null neurons. By 4 days AE, *dfmr1*-null neurons display fewer axon branches [length: 72.9±29.7 μm (*n*=22), *P*=0.01 relative to eclosion (Fig. 10A); number: 7.2±3.1 (*n*=22), *P*=0.002 relative to eclosion; Fig. 10B]. Indeed, over-pruning in *dfmr1*-null neurons was evident in the reduction of small processes (<5 μm) compared with controls [control: 5.4±2.7 (*n*=24); *dfmr1*: 3.5±1.9 (*n*=22), *P*=0.01]. Thus, there is an early-use period of pruning whose timing is dependent on dFMRP.

Sensory deprivation revealed that dFMRP function is activity dependent. In control SD neurons, there was a significant increase in axon branch number [4d control: 9.0±3.3 (*n*=24); 4d SD: 12.1±3.8 (*n*=20), *P*=0.028; Fig. 10B], with a non-significant tendency towards greater length [4d control: 68.9±27.8 μm (*n*=24); 4d SD: 82.7±21.2 μm (*n*=20), *P*=0.17]. In *dfmr1*-null SD neurons, there was a more pronounced increase in branch number [4d: 7.23±3.1 (*n*=22); 4d SD: 13.4±3.5 (*n*=24), *P*<0.001; Fig. 10B], and branch length was also increased [4d: 72.9±29.7 μm (*n*=22); 4d SD: 107.3±27.8 μm (*n*=24), *P*<0.001; Fig. 10A]. Acute stimulation following SD rearing was unable to induce pruning in either *dfmr1*-null or control neurons (Fig. 10). Thus, sensory input activity strongly influences both the timing and extent of the dFMRP-dependent pruning of axonal processes.

DISCUSSION

It remains unknown whether Fragile X Syndrome (FraX) is a disease of development, a disease of plasticity, or a combination of both, although these possibilities give rise to entirely different strategies for therapeutic intervention. Most recent FraX research has focused on acute defects in synaptic plasticity, with only minimal attention

given to developmental dysfunction. A key concept is that a restricted function during development may well be reflected in impaired plasticity at maturity, and thus the timing of dysfunction does not necessarily correspond to the period of functional requirement. Although mammalian studies have indicated peak FMRP levels during early postnatal development (Khandjian et al., 1995; Lu et al., 2004; Singh et al., 2007; Wang et al., 2004), very little work has characterized the temporal requirements of FMRP. We therefore employed the *Drosophila* FraX model to analyze dFMRP-dependent processes in neuronal development. Our initial findings highlighted roles for dFMRP in (1) late stages of brain development and (2) very early-use circuit refinement, and we therefore focused on these mechanisms.

In the absence of dFMRP, elevated levels of total RNA/protein are evident during a restricted period of late pupal brain development, with the protein increase persisting into an early-use refinement period (Fig. 1). These increases are transient and disappear in the mature brain thereby defining a limited developmental window of dFMRP function. The increase in protein is predicted as FMRP/dFMRP negatively regulates translation (Khandjian et al., 2004; Laggerbauer et al., 2001; Li et al., 2001; Qin et al., 2005; Sung et al., 2003). The elevated RNA is more surprising. dFMRP/FMRP can both negatively and positively regulate mRNA stability (Xu et al., 2004; Zalfa et al., 2007; Zhang et al., 2007), and, therefore, dFMRP may have a developmentally-restricted role primarily as a negative regulator of mRNA stability. Alternatively, the RNA increase may be caused by elevated transcription, via an uncharacterized direct or indirect transcriptional inhibition function of dFMRP. Because the increase in total protein/RNA is not biased towards selected dFMRP targets, these results suggest globally upregulated transcription/translation in the *dfmr1* mutant brain during a restricted window of late maturation and early-use refinement.

During brain development, *dfmr1* mRNA and dFMRP protein levels tightly correlate with the above changes, but, surprisingly, *dfmr1* mRNA levels inversely correlate with dFMRP protein levels in the mature brain (Fig. 2). By 4 days AE, *dfmr1* mRNA levels rise to levels nearly as high as those present during development, but dFMRP protein is maintained at a basal level in the mature brain. This change strongly suggests a distinct switch in dFMRP regulation, in which transcription and translation become uncoupled. Because dFMRP/FMRP represses the translation of its own mRNA (Ashley, Jr et al., 1993; Brown et al., 1998; Ceman et al., 1999; Schaeffer et al., 2001; Sung et al., 2000), it is tempting to speculate that this negative-feedback mechanism specifically regulates dFMRP in the mature brain. FMRP modulates synaptic plasticity at maturity, as evidenced by decreased LTP and enhanced LTD in *fmr1* knock-out (KO) mice (Hou et al., 2006; Huber et al., 2002; Koekkoek et al., 2005; Larson et al., 2005; Li et al., 2002; Wilson and Cox, 2007; Zhao et al., 2005). Consistent with such a mature function, elevated total protein levels are once again evident in the fully mature *dfmr1*-null brain (Fig. 1). A similar increase in cerebral protein synthesis occurs in adult *fmr1*-KO mice (Qin et al., 2005). Together, these data suggest that a switch in dFMRP/FMRP regulation defines separate windows of function in development versus maturity.

It was crucial to determine whether dFMRP function correlated with its developmental expression profile. A known dFMRP target is *chickadee*/*profilin*; dFMRP binds *chickadee* mRNA and negatively regulates its translation (Reeve et al., 2005). Importantly, the dynamics of *chickadee* misregulation in the *dfmr1*-null brain indicate that the dFMRP functional requirement mirrors its developmental expression profile (Fig. 3). Chickadee expression normally peaks during late-stage brain development (P4), and it is during this development window, and shortly following, that overexpression is manifested in the *dfmr1*-null brain. Generally, the increase in *chickadee* transcripts parallels the increase in protein, suggesting that dFMRP regulation may be at the level of the mRNA, for example, by affecting mRNA stability. dFMRP reportedly interacts with miRNA machinery to control mRNA levels of the sodium channel Pickpocket1 (Xu et al., 2004). A similar mechanism for *chickadee* regulation would be consistent with our results. Interestingly, the increase in Chickadee protein levels coincides with the period of use-dependent neural circuit refinement at eclosion. At least one dFMRP/FMRP target mRNA, *futsch* (*MAP1B*), is regulated specifically at postnatal day 10 in *fmr1*-KO mice (Lu et al., 2004). These new insights suggest it will be vital to ascertain the developmental expression of all putative FMRP targets in the context of these distinct windows of regulation in order to validate in vivo functions.

During the peak period of dFMRP expression, there are two phases of dFMRP-dependent axon maturation. During late pupal development, dFMRP inhibits axon elongation, with *dfmr1*-null neurons exhibiting branches 25% longer than controls (Fig. 4). This function is restricted to very late stages (P4), with no differences in branch length or number being observed earlier (P3). Immediately upon eclosion, dFMRP is required for use-dependent pruning, causing a decrease in both axon branch length and number (Fig. 4). Pruning is most evident in the smallest presynaptic branches (<5 μm) and occurs quickly (hours) following the onset of adult activity. Targeted overexpression of dFMRP causes inverse defects in both phases of dFMRP requirement (Fig. 5). Axon undergrowth is apparent early (P3) and axons fail to grow later (P4). Axon branches present in these neurons are short, filipodial-like structures, and, at eclosion, there is excessive pruning to result in ~30% fewer branches than in P4 and ~3 times fewer branches than in controls (Fig. 5). Thus, both axonogenesis and axon branch pruning are bidirectionally modified by inverse changes in dFMRP expression.

Blocking sensory input activity maintains dFMRP in its early development regulative state, with a correlative reduction in both *dfmr1* mRNA and dFMRP protein (Fig. 6). Both olfactory (*Or83b*) and phototransduction (*ninaE*) mutants (Larsson et al., 2004; O'Tousa et al., 1985) similarly suppress dFMRP levels, indicating that these two primary modes of brain sensory input positively drive dFMRP expression (Fig. 7). Similarly, mammalian FMRP expression is elevated following activity stimulation by both environmental enrichment and mGluR signaling activation (Gabel et al., 2004; Hou et al., 2006; Irwin et al., 2005; Todd and Mack, 2000; Todd et al., 2003; Valentine et al., 2000; Weiler et al., 1997). Blocking mGluR activity in *Drosophila* and mice can rescue some *dfmr1* defects, including impaired learning and memory (McBride et al., 2005; Yan et al., 2005; Pan et al., 2008). From these similar findings, it is tempting to suggest that dFMRP/FMRP may function downstream of mGluR signaling activity, perhaps differentially in development versus maturity. Importantly, both *Or83b* and *ninaE* sensory mutants cause elevation of *chickadee*/*profilin* at the same time dFMRP is suppressed (Fig. 7). This finding is consistent with activity-dependent regulation of dFMRP to regulate *chickadee*/*profilin* expression.

This study shows for the first time that *Drosophila* neurons undergo activity-dependent pruning coincident with the onset of use. In the absence of dFMRP, pruning does not occur during the normal developmental window (Fig. 4). Indeed, blocking sensory input activity leads to further increases in the axon branch number and

length in *dfmr1*-null neurons. Moreover, at maturity, sensory stimulation following sensory deprivation does not induce pruning, probably because the dFMRP level has fallen too low. We hypothesize that there is a threshold of dFMRP required for efficient activity-dependent pruning during the early-use period, which is normally defined by the window of high dFMRP expression. Reinstated sensory stimulation following sensory deprivation does cause a significant dFMRP-dependent increase in the number of long axon branches (>10 μm; Fig. 8). These data are consistent with the need for high dFMRP expression to both limit axonal growth and mediate the early-use refinement of circuits. Importantly, we confirmed, by using targeted expression of the exogenous light-gated channelrhodopsin-2 channel (Schroll et al., 2006), that neuronal activation bidirectionally drives the pruning process. Light-driven activation of CHR2 channels induces pruning of the same small (<5 μm) axonal processes that aberrantly persist in the *dfmr1*-null brain (Fig. 9). As predicted, the induced pruning process fails to occur in the absence of dFMRP.

Delayed pruning eventually occurs in *dfmr1*-null neurons to ultimately rescue the overbranching defect present in younger animals. A similar transient elongation of dendritic spines occurs in young postnatal *Fmr1*-KO mice, although a secondary overgrowth phenotype may appear months later in adult animals (Galvez and Greenough, 2005; Nimchinsky et al., 2001). In *Drosophila*, the delayed axon pruning in *dfmr1*-null neurons actually goes too far, resulting in reduced neuronal complexity in mature adult animals (Fig. 10). The small presynaptic branches (<5 μm) are reduced 35% in *dfmr1*-null neurons compared with controls at 4 days. Because pruning normally occurs very rapidly (<3 hours after eclosion), coincident with initial use, it is likely that the pruning process is strictly controlled for that developmental time. By delaying pruning in the absence of dFMRP, it appears that other factors that buffer the extent of process elimination fail to provide adequate regulation of the mechanism. Indeed, this mitigation may be a function of dFMRP itself, as dFMRP levels drop drastically immediately following the normal pruning window. FMRP potentially regulates many proteins involved in a diverse set of functions (Brown et al., 2001; Miyashiro et al., 2003; Zhang et al., 2005). Understanding the developmental regulation of proteins that associate with FMRP and FMRP target mRNAs will be crucial to unraveling the underlying pruning mechanisms of activity-dependent neural circuit refinement.

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