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# The Fragile X Mental Retardation Protein in Circadian Rhythmicity and Memory Consolidation

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**Abstract** The control of new protein synthesis provides a means to locally regulate the availability of synaptic components necessary for dynamic neuronal processes. The fragile X mental retardation protein (FMRP), an RNA-binding translational regulator, is a key player mediating appropriate synaptic protein synthesis in response to neuronal activity levels. Loss of FMRP causes fragile X syndrome (FraX), the most commonly inherited form of mental retardation and autism spectrum disorders. FraX-associated translational dysregulation causes wide-ranging neurological deficits including severe impairments of biological rhythms, learning processes, and memory consolidation. Dysfunction in cytoskeletal regulation and synaptic scaffolding disrupts neuronal architecture and functional synaptic connectivity. The understanding of this devastating disease and the implementation of meaningful treatment strategies require a thorough exploration of the temporal and spatial requirements for FMRP in establishing and maintaining neural circuit function.

**Keywords** Fragile X syndrome · Learning · Memory · Circadian rhythm · Mushroom body · Clock circuit · Neuron · Axon · Dendrite · Synapse · *Drosophila*

## Introduction

Neurons exhibit extreme polarity and inherent architectural complexity, including extensive dendritic arbors and lengthy axonal projections. Within this context, efficient resource allocation dictates subdomain compartmentalization of protein synthesis to provide for rapid and dynamic responses to cellular demands. Local translation control facilitates immediate and enduring regulation of synaptic connections, with respect to structure and transmission strength, both during early synaptogenesis and later connectivity modulation in experience-dependent plasticity. At the behavioral output level, such regulation would predictably manifest most readily in association with processes that require expeditious and stringent molecular management. The daily cycling of components enabling circadian rhythms and the neuronal reinforcement underlying memory formation are two such examples. Regulated *de novo* protein synthesis at synapses could provide the critical and consistent short- and long-term molecular plasticity required to enable these events.

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## Local Translational Control, FMRP, and FraX

The utilization of such translational control is clearly evident when this regulation becomes compromised, as in fragile X syndrome (FraX). FraX is caused by the silencing of a single gene, *fragile X mental retardation 1* (*FMR1*) [1], causing the most common heritable form of mental retardation and autism spectrum disorders [2–10]. The *FMR1* product (fragile X mental retardation protein (FMRP)) is a messenger RNA (mRNA)-binding polysome-associated protein that acts as a negative regulator of protein translation [11–17], facilitates mRNA trafficking [18, 19], and influences mRNA stability [20, 21]. FMRP may also bind noncoding adaptor RNAs [13, 21, 22], microRNAs (miRNAs) [23–25], and components of the RNA-induced silencing complex (RISC) [26–28]. Further, the expression and function of FMRP are regulated

downstream of synaptic activity [15, 29–35] and are closely implicated in the activity-dependent control of synaptic connections [15, 36–40]. As such a multifaceted pivotal molecular player, the resultant clinical manifestations upon the loss of FMRP often include delayed and depressed developmental trajectories [3, 9, 41], deficits in short-term working memory [42–45], disordered sleep [46, 47], seizures [48–54], and the canonical cytological presentation of morphologically aberrant synaptic connections, i.e., elongated and immature dendritic spines [55–58].

FMRP contains a nonclassical nuclear localization sequence (NLS) and a leucine-rich nuclear export sequence (NES) [59], suggesting it shuttles to and from the nucleus [60], which may therefore be the site of its association with target mRNAs. In support of this mechanism, FMRP had been shown to interact with a distinct mRNA nuclear export factor, NXF2, but not with its ortholog NXF1, the ubiquitously expressed principal mRNA nuclear export receptor, in mouse germ line cells and hippocampal neurons [61]. A recent contradictory report using cultured Cos-7 cells indicates that enhanced green fluorescent protein (EGFP)-FMRP is largely retained within the nucleus upon NXF1 depletion and that EGFP-FMRP and NXF1 do interact in an RNA-dependent fashion [62]. However, FMRP and NXF2 appear to work in concert to destabilize *Nxf1* mRNA in cultured mouse neuroblast N2a cells [20], perhaps providing a mechanism by which FMRP mediates the preferential exploitation of NXF2 as an alternative export factor in neurons. FMRP also has a number of well-characterized mRNA-binding motifs, including two hnRNP K-homology (KH) domains, an arginine- and glycine-rich domain (RGG box), and an additional N terminus RNA-binding activity [63–67].

## FMRP Target Identification

FMRP was initially proposed to bind ~4% of human fetal brain mRNAs *in vitro* [63]. In the subsequent 15 years, a myriad of approaches has been employed to elucidate the mechanisms mediating these interactions, identify specific target mRNAs, and validate their biological relevance and specificity. *In vitro* RNA selection demonstrated that the FMRP RGG box serves as a sequence-specific binding domain engaging intramolecular G quartets within its target mRNAs, with UniGene database mining suggesting 31 potential mRNA targets including *microtubule-associated protein 1B* (*MAP1B*) and *neuronal acidic protein 22* [68]. In murine microarray screens, FMRP associated with ~4% (432:11,067) of total brain mRNAs, including the translational regulator *Pumilio* and the apparent circadian rhythm protein *SCOP* [69]. Additional polysome association shift assays comparing normal and FraX patient lymphoblastoid cells revealed that ~2% of mRNAs exhibit altered translational profiles; interestingly, both increases and decreases were observed suggesting FMRP may exert positive and negative translational regulation [69]. Complementary DNA (cDNA) systematic evolution of ligands by exponential enrichment identified U-rich regions as another potential FMRP mRNA recognition motif [70]. Antibody-positioned RNA amplification initially identified 223 putative FMRP-associated mRNA targets in a neuroarray screen [71], with secondary confirmation of 60% of these by filter binding and UV cross-linking assays, including the *GABA-A receptor δ-subunit* and *β-adaptin*. *Drosophila* microarray screens yielded 83 target mRNAs, one of which encoded the cytoskeletal protein lethal giant larvae [72]. Human whole-genome microarray analysis has similarly implicated 90 targets [73]. A proteomic approach combining two-dimensional difference gel electrophoresis and mass spectrometry indicated 24 altered protein species, including phenylalanine hydroxylase involved in monoamine (dopamine) synthesis [74]. A yeast three-hybrid screen using a human fetal hippocampal cDNA library yielded 22 target mRNAs, 18 of which were validated *in vitro* via gel retardation assays, including a key enzyme in cellular redox processes, *Thioredoxin reductase 1* [75]. The most recent investigative panel examined quantitative proteomic changes in synaptic proteins in *Fmr1* knockout (KO) mice by stable isotope labeling by amino acids in cell culture combined with multidimensional protein identification techniques [76], revealing >100 proteins that are either upregulated or downregulated, such as the RNA-binding protein FUS and the catenin-like protein ARVCF, respectively.

Surprisingly, there is little overlap in the candidates identified with these different approaches, perhaps suggesting developmental stage and/or tissue specificity associated with particular cohorts of FMRP targets. Thus, the exact number and formal identity of FMRP mRNA targets remain uncertain. Many of these reported targets must be considered candidates only, awaiting full verification, and determining the scope of FMRP function in the regulation of mRNA targets remains one of the most pressing needs for the field. However, as these candidate targets range from synaptic constituents, cytoskeletal and adaptor proteins, cell surface receptors, adhesion molecules, and extracellular matrix (ECM) components to modulators of gene and protein expression and intracellular signaling molecules, the fundamental importance of FMRP comes into sharp focus. This review will discuss the molecular foundations of current FMRP hypotheses, including the modulation of components influencing neuronal architecture, the direct role recently illustrated in mRNA trafficking, and new insights into the bidirectional regulation of synaptic excitability. This review will then primarily focus on highlighting the exciting new findings on the role of FMRP

in circadian circuits to regulate daily activity rhythms and the role of FMRP in learning and memory circuits to enable plasticity and the ability to remember.

## Molecular Mechanisms of FMRP Function

FMRP incorporates into large messenger ribonucleoprotein particles (mRNPs) often associated with actively translating polysomes [77–81]. Neuronal FMRP granules containing ribosomes, ribosomal RNAs, and target mRNAs localize to both developing axons and dendrites, including filopodia, dendritic spines, and growth cones [22, 81, 82]. Despite the suggestion of bidirectional translation control [69, 76], FMRP has only been shown to suppress protein synthesis [11–17, 34, 35]. Therefore, in the absence of FMRP, its targets appear constitutively elevated and thus removed from regulative control. As most of the well-characterized targets of FMRP regulation are associated with cytoskeletal and synaptic organization, defective neuronal structural development and aberrant synapse formation ensue.

### FMRP and Cytoskeletal Regulation

The role of FMRP as a negative translation regulator was first established *in vivo* in the *Drosophila* system, for the first identified *in vivo* target Futsch, the *Drosophila* homolog of MAP1B (Fig. 1) [83]. *Drosophila* FMRP (dFMRP) associates directly with *futsch* mRNA, and Futsch protein level in the brain is both elevated in the absence of dFMRP and reduced upon dFMRP overexpression. Futsch regulates microtubule cytoskeleton stability to influence neuronal growth at the axonal, dendritic, and synaptic levels [84–87]. Critically, *dfmr1*-null synaptic phenotypes are compensated for by concomitant reduction of *futsch* [83] to mimic levels present in the wild-type condition. Recently, it was shown that reintroduction of neuronal dFMRP in the *dfmr1* mutant restores Futsch cytoskeletal organization at the synapse [88]. As a consequence of these *Drosophila* studies, MAP1B was subsequently shown to be similarly regulated by FMRP in mammalian neurons [17]. In *Fmr1*-null mice, the abnormally elevated levels of MAP1B also yield aberrant enhanced microtubule stability [17].

Independently, the actin cytoskeleton is also subject to FMRP regulation (Fig. 1). For example, dFMRP negatively regulates the actin-binding protein Chickadee [15, 89], the *Drosophila* profilin homolog [90]. *chickadee* mRNA associates with dFMRP mRNPs, and Chickadee protein level is elevated in the *Drosophila* FraX model [15, 89]. As profilin influences actin dynamics and stability [91], its misregulation in *dfmr1* mutants yields significant neuronal structural defects [89]. In addition, through regulation of

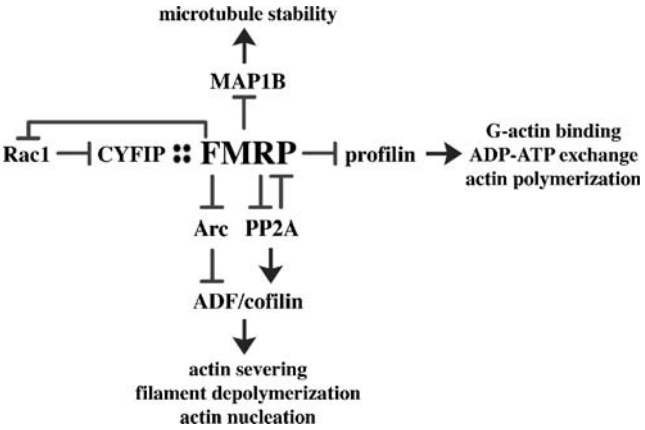


**Fig. 1** FMRP and the cytoskeleton. The interactions between FMRP and both the microtubule and actin cytoskeleton elements are modeled. MAP1B, which impinges upon microtubule stability, is negatively regulated by FMRP [17, 83]. This interaction normally provides for regulated synaptic deposition and growth. Regarding FMRP and the actin cytoskeleton, one known pathway involves the small GTPase Rac1 [92], which antagonizes the formation of the FMRP::CYFIP complex [93, 147]. These components then negatively influence profilin functionality [89], which serves to promote actin filament polymerization. In the absence of FMRP, there is no attenuation of the resulting cellular protrusion causing neuronal overgrowth. FMRP also potentially alters actin dynamics by influencing the activation state of ADF/cofilin, which mediates filament severing and depolymerization, while also capable of nucleation. Though more often discussed in relation to AMPA receptor internalization in concert with FMRP and eEF2K [106], Arc is also linked to facilitating cofilin modifications that allow cytoskeletal expansion [245]. As FMRP negatively regulates Arc translation, this could contribute to appropriate neuronal structuring. FMRP also negatively regulates the cofilin phosphatase, PP2A [96], which in turn can mediate dephosphorylation of FMRP creating a negative feedback loop [130, 131]

the small guanosine triphosphatase (GTPase) Rac1 [92] and CYFIP/Sra-1 (cytoplasmic FMRP-interacting protein) [93, 94], dFMRP plays a key role in modulating the actin cytoskeleton during neuronal morphogenesis (Fig. 1). CYFIP interacts both biochemically and genetically with dRac1 and dFMRP in *Drosophila*, acting as a dRac1 effector antagonizing dFMRP [93]. Studies in murine fibroblasts subsequently demonstrated mechanistic links between FMRP and actin depolymerizing factor (ADF)/cofilin, a mediator of Rac1-dependent F-actin nucleation, severing, and depolymerization [95]. The phosphoserine/threonine phosphatase 2A (PP2A) dephosphorylates, and thus activates, phospho-cofilin [96, 97]. FMRP binds the 5′-untranslated region (UTR) of the *PP2A catalytic subunit* (*pp2ac*$\beta$) mRNA likely providing negative translational regulation [96]. Thus, upon the loss of FMRP, PP2A is elevated; phospho-cofilin is depressed, and actin dynamics are enhanced leading to morphological disruption in neuronal development (Fig. 1). In agreement, neuronal overexpression of the *Drosophila* PP2A β' regulatory subunit (*well rounded*) causes synaptic terminal overgrowth [98].

## FMRP Interplay with Synaptic Components, CAMs, and ECM

In addition to cytoskeleton regulation, FMRP exerts a direct synaptic influence by regulating postsynaptic density protein 95 (PSD-95), a PDZ domain scaffold and member of the membrane-associated guanylate kinase family of adaptors [99]. FMRP has been shown to bind the 3′-UTR of *PSD-95* conferring enhanced message stability [100]. PSD-95 is rapidly translated upon metabotropic glutamate receptor (mGluR) stimulation with the agonist S-3,5-dihydroxyphenylglycine (DHPG), and this response is lost in FMRP-deficient neurons [35, 101]. Similarly, mGluR-stimulated regulation of *calcium/calmodulin-dependent kinase IIα* (*CaMKIIα*) mRNA levels is altered in the absence of FMRP [35, 102]. In *Drosophila*, CaMKII directly influences the PSD-95 homolog discs large (DLG), such that CaMKII-dependent DLG phosphorylation regulates the association of DLG with the synaptic complex [103]. This regulation plays a critical role in the control of synaptic growth and postsynaptic domain composition via interaction with the position-specific integrin receptors at the synaptic cleft interface [104]. Thus, it is clear that FMRP can moderate the regulated formation and molecular composition of the postsynaptic domain. One outcome of FMRP deficiency is excessive internalization of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptor subunits [105], mediated via the coincident deregulation of the FMRP target activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) [106, 107].

Outside the cell, FMRP may influence cell adhesion molecules (CAMs) as well as components of the ECM. Proteomic analyses have revealed quantitative changes in neurexin-1α, neuroligin-2, cadherin-13, and neural cell adhesion molecule in primary neurons derived from *Fmr1*-null mice [76]. However, there was no attempt to examine potentially associated alterations in mRNA levels. Further testing will be required to determine whether any of these are direct FMRP targets, as these deviations may well be downstream secondary consequences of the loss of FMRP. Interestingly, it was recently reported that treatment with a tetracycline analog, minocycline, can promote dendritic spine maturation and behavioral reparation in *Fmr1*-null mice [108]. This study suggested that minocycline mediated inhibition of matrix metalloproteinase 9, which is elevated in the *Fmr1* mutant and could lead to excessive cleavage of stabilizing cell surface or ECM components. Thus, FMRP potentially acts at the ECM/transmembrane receptor synaptic interface, on membrane-associated scaffolds, and on the regulation of cytoskeletal dynamics.

## The Role of FMRP in RNA Transport

In addition to regulating mRNA translation, FMRP may regulate the local proteomic landscape via transport and delivery of its target mRNAs to specialized sites of critical need. FMRP appears to be involved in mRNA trafficking within neurons. The neuronal translocation of FMRP granules is bidirectional and reliant upon kinesin- and dynein-associated microtubule cytoskeleton transport [18, 19, 81, 109–112]. Two recent studies have demonstrated a direct role for FMRP in activity-dependent mRNA transport [18, 19]. A study in the *Drosophila* FraX model used a genetically encoded MS2 phage protein tracking method [113, 114] to examine mRNA transport of *chickadee* and *CG9293* transcripts in primary cultured neurons; *CG9293* is a homolog to human inhibitor of growth (*ING1*) implicated in carcinoma development [19]. In *dfmr1*-null neurons, mRNA granules exhibited depressed net distances traveled and reduced overall mRNA mobility, suggesting a role for dFMRP in facilitating mRNA trafficking. Fluorescence recovery after photobleaching revealed that dFMRP enhances mRNA mobility in a dosage-dependent manner [19]. A coincident study in mice using fluorescence *in situ* hybridization showed altered mRNA dendritic localization in response to mGluR stimulation with the agonist DHPG in *Fmr1*-null hippocampal cultures, including *MAP1B*, *CaMKIIα*, *SAPAP4*, and *GABA-A receptor δ* transcripts [18]. This work suggests an explicit role for FMRP in activity-dependent mRNA trafficking near synapses. Live cell imaging using a GFP-MS2-*CaMKIIα* mRNA reporter [114] showed that mRNA particle motility depends upon the presence of FMRP [18]. Additional experiments revealed that mRNA motility is largely mediated through interaction of FMRP with kinesin light chain (KLC), although previous work in *Drosophila* has implicated the heavy chains of either kinesin-1 or cytoplasmic dynein as being responsible for granule translocation, with RNAi-mediated KLC knockdown having no effect [110]. In contrast to these studies, it has also been reported that *PSD-95* is localized to the synapse independently of FMRP but that FMRP confers message stability [100]. Thus, FMRP may exert differential modes of influence on specific target mRNAs. Taken together, these data provide an attractive mechanistic view as to how FMRP may fundamentally regulate its target mRNAs to modulate synaptic structure and function.

## The Gq Theory of FraX

Early *in vitro* studies revealed that FMRP is translated upon the protein synthesis induction mediated by mGluR activation [29, 115]. Subsequent examinations revealed that

FMRP upregulation can be facilitated *in vivo* by enhancing sensory input. For example, complex environmental exposure and motor skill training elevate FMRP levels in mice [31, 116]. Unilateral whisker stimulation also increased FMRP expression in synaptosomal and polysomal fractions derived from the somatosensory cortex [117]. This upregulation is at least partially dependent upon mGluR activation, as pharmacological blockade using 1-aminoindan-1,5-dicarboxylic acid absolves the increased expression, although *N*-methyl-D-aspartic acid receptor (NMDAR) inhibition is also effective [101]. Visual experience likewise elevates FMRP levels but is NMDAR and not mGluR dependent [118]. Experiments in the *Drosophila* model illustrated that sensory input deprivation mediates the anticipated inverse effect on dFMRP levels [15]. The presentation of depressed dFMRP levels in sensory-deprived wild-type controls or sensory transduction mutants (*ninaE* or *Or83b*) revealed that visual and olfactory modalities positively regulate dFMRP [15]. Conversely, known dFMRP targets are negatively regulated by sensory input activity, consistent with the repressive function of dFMRP. Thus, multiple sensory input pathways clearly lead to neuronal activation and subsequent FMRP regulation.

A now well-established mechanistic hypothesis of FraX suggests that FMRP functions downstream of mGluR signaling activity at the synapse [40, 119], which plays key roles in excitatory neurotransmission, neuronal development, and synaptic plasticity [120]. This "mGluR theory of FraX," which implicates elevated mGluR signaling as a cause of FraX-related symptoms, is based on the observation that group I mGluR1/5 signaling via Gq-mediated transduction stimulates the translational machinery at synapses and causes upregulation of FMRP levels [29, 30, 33–35, 115, 121]. In addition, increased synaptic protein synthesis is obligate for a type of plasticity known as mGluR-dependent long-term depression (LTD), important in activity-dependent synapse elimination [122]. Preparations from *Fmr1* KO mice do not display mGluR-triggered polysome assembly or induced protein synthesis [33–35, 101, 106]. In fact, the null mutant mice surprisingly present increased mGluR-triggered LTD [38, 39]. In addition, mGluR5 antagonism with 2-methyl-6-phenylethynyl-pyridine (MPEP) in *Fmr1* KO mice rescues audiogenic seizure susceptibility, open-field exploratory hyperactivity, and prepulse inhibition of startle phenotypes [123, 124]. Similarly, genetic reduction of mGluR5 signaling in the *Fmr1*-null background rescues altered ocular dominance plasticity, cortical neuron dendritic spine density, increased basal protein synthesis, inhibitory avoidance extinction, audiogenic seizure sensitivity, and accelerated body growth [125]. Thus, FMRP acts as a negative regulator of the mGluR pathway, influencing a range of signaling pathways, including those of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K), which likely coordinate the facilitation of early eukaryotic translation initiation factor 4E (eIF4E) release stimulating synaptic protein translation [126–128]. Recent evidence implicates an FMRP interactor, CYFIP1/Sra1, in directly binding eIF4E through a 4E-BP-like translational inhibitor domain, with activity-dependent CYFIP1 dissociation from eIF4E at synapses resulting in protein synthesis [129]. This bidirectional regulation is controlled via the phosphorylation state of FMRP. Rapid FMRP dephosphorylation is mediated by the phosphatase PP2A and is temporally associated with transient elevations in FMRP target mRNA translation profiles [130]. However, prolonged mGluR activation yields mammalian target of rapamycin (mTOR)-mediated PP2A suppression and rephosphorylation of FMRP [130]. Opposing PP2A functionality, ribosomal protein S6 kinase phosphorylates FMRP upon activation of ERK and mTOR, thereby reestablishing translational suppression and returning the system to its steady state [131].

Although the role of mGluR in FraX models is well documented, it is clear from the symptoms of FraX patients that defects are not limited to glutamatergic circuits. Rather, behavioral abnormalities are associated with multiple neurotransmitter systems, including fast cholinergic transmission as well as slow modulatory transmission mediated by biogenic amines (e.g., dopamine) and neuropeptides [74, 132]. This suggests that FMRP is not simply responding to mGluR signaling but likely acting in a more general feedback loop linking neurotransmitter signaling via Gq-coupled receptors for a range of neurotransmitters and neuromodulators. This "Gq theory of FraX" was initially postulated based on the work of Huber and colleagues [133] wherein the influence of $M_1$ muscarinic acetylcholine receptor (mAChR) activation on LTD was explored. Pharmacologic mAChR stimulation using the agonist carbachol or synaptic stimulation inducing intrinsic acetylcholine release using paired-pulse low-frequency stimulation (PP-LFS) revealed protein-synthesis-dependent LTD, associated AMPA receptor (AMPAR) internalization, elevated levels of FMRP, and coordinated increases in the FMRP targets elongation factor 1α and αCaMKII. Further, this mAChR-mediated LTD was enhanced in *Fmr1* KO mice due to LTD-associated proteins being constitutively elevated in the absence of translationally suppressive FMRP activity, effectively uncoupling LTD induction from requisite protein synthesis [133].

These results paired with the extensive mGluR investigations indicate the convergence of various Gq-coupled cholinergic and glutamatergic receptors upon mediating synaptic plasticity, expanding the mechanistic supposition

to understanding signaling alterations in FraX from the "mGluR theory" to the more inclusive "Gq theory." In this expanded theory, input activity drives neurotransmitter release, which is tracked and monitored by circuit-appropriate Gq-linked receptors. It is the response of the associated signal transduction cascades that convergently fine-tunes the level and function of FMRP, controlling protein synthesis to regulate synaptic structure and function relative to circuit activity.

## Exploiting the *Drosophila* FraX Model

FraX genetic animal models are, of course, indispensable [83, 134–136]. In particular, with its amenability to genetic manipulation and accessible simplified neuronal circuitry, the *Drosophila* FraX model, generated through mutational analysis of the sole *Drosophila fmr1* gene [83, 137–139], has proven to be an invaluable experimental system. dFMRP harbors the full complement of known and proposed mammalian functional domains, including RNA-binding motifs (2 KH domains, 1 RGG box), NLS and NES, 60S ribosomal subunit interaction domain, protein–protein interaction domains (coiled-coil and PPiD), and a kinase phosphorylatable PhD domain [59, 63, 64, 83, 140]. Phenotypically, null *dfmr1* mutants accurately mimic a host of FraX clinical symptoms including irregular circadian motor activity [137, 139, 141, 142], impaired social interaction (e.g., courtship ritual [137, 143]), reduced learning and severe loss of protein synthesis-dependent long-term memory [32], overgrown and overbranched neuronal structures in numerous neural circuits [15, 83, 88, 89, 92, 137–139, 144–147], and compromised neuronal and synaptic function [36, 83, 88].

### Testing the Mechanistic Theories of FraX

One important use of the *Drosophila* FraX model has been to systematically test the "mGluR theory." The *Drosophila* genome encodes two potential mGluRs (DmGluRA and DmGluRB), but only DmGluRA binds glutamate and is therefore the sole functional receptor [148, 149]. In contrast, mammals possess eight mGluRs classified into three groups [150]; thus, *Drosophila* represents a greatly simplified system. DmGluRA localizes both presynaptically and postsynaptically and regulates both synaptic structure and activity-dependent function [146, 151]. Importantly, dFMRP and DmGluRA display mutual negative feedback such that in *dfmr1* mutants DmGluRA is upregulated and in *DmGluRA* mutants dFMRP is upregulated [146]. Structurally, at the glutamatergic neuromuscular junction (NMJ), *dfmr1* deficiency yields enlarged synaptic terminals with excessive branching, elevated synaptic bouton numbers, accumulation of miniature/satellite boutons, altered cytoskeletal organization, and increases in synaptic vesicle density, active zone clustering, and docking [83, 88, 146]. Mutations in *DmGluRA* lead to more modest defects in NMJ architecture including decreased synaptic bouton numbers and increased mean bouton size [146, 151]. mGluR attenuation in the *dfmr1* nulls by either coincident *DmGluRA* mutation (*dfmr1*;*DmGluRA* double-null mutants) or antagonism with MPEP restores normal NMJ branching, synaptic vesicle density, and vesicle active zone association [146]. The rescue provided is partial; however, this further supports the "Gq theory" suggesting that dFMRP function is modulated by several impinging modulatory pathways. Within the olfactory learning and memory center of the *Drosophila* brain, i.e., the mushroom body (MB), neuronal morphologies are negatively regulated by dFMRP, with *dfmr1* mutation causing excessive axonal and dendritic elaboration, more numerous synaptic connections, and elevated synaptic vesicle density [15, 138, 143, 144, 146]. Pharmacologically reducing DmGluRA signaling mitigates these MB synaptic growth defects [143, 146].

### Exploring FraX-associated Synaptic Hyperexcitability

Functionally, basal synaptic transmission properties of *dfmr1*, *DmGluRA*, and *dfmr1*;*DmGluRA* double-null mutants are relatively normal [36, 83, 151], although *dfmr1* nulls exhibit an increased frequency of vesicle fusion events and enhanced exocytic vesicle cycling [83, 88]. However, under more challenging high-frequency stimulation (HFS) paradigms, striking alterations are unveiled. Null *dfmr1* mutants exhibit properties of synaptic hyperexcitability illustrated by multiple discrete excitatory junctional current events in response to individual stimuli and periodic amplitude cycling during HFS [36]. These defects are partially restored toward wild type in *dfmr1*;*DmGluRA* double-null mutants. In support of this dFMRP-deficient hyperexcitable state, recent work in the mouse FraX model revealed decreases in unitary connective frequency and strength resulting in a substantial decrement in excitatory drive onto local fast-spiking inhibitory neurons [37]. Further, the excitatory neurons maintain differential membrane properties yielding increased action potential firing and concomitantly lowered evoked threshold in the absence of FMRP. These results collectively indicate FraX-associated neural hyperexcitability, not only at the single-cell level but also within entire functional circuits. This was confirmed upon the examination of periodic persistent activity, or "UP" states, which were twofold increased in duration in the *Fmr1* null as compared to control [37]. Considering other synaptic properties, an unexpected synergism in the *dfmr1*;*DmGluRA* double-mutant results in enhanced short-term facilitation, compared to either single mutant [36, 151]. In *DmGluRA* nulls, extended HFS heightens the

prolonged augmentation response, often associated with aberrant asynchronous breakaway responses characteristic of premature long-term facilitation (LTF) [151], with *dfmr1*; *DmGluRA* comutation slowing onset kinetics [36]. Null *DmGluRA* mutants also display enormously elevated posttetanic potentiation (PTP) after prolonged HFS [151], which is effectively rescued by coremoval of *dfmr1* [36]. These data suggest a complex coordination of mGluR signaling on FMRP, mediating at least a partial feedback loop involved in regulative maintenance of activity-dependent plasticity.

In the postsynaptic domain, dFMRP and DmGluRA cooperate in the specification of ionotropic glutamate receptors (iGluRs) [145]. At the NMJ, two iGluR classes are resident, both harboring the common IIC, IID, and IIE subunits paired with a variable IIA (A-class iGluR) or IIB (B-class iGluR) subunit [152, 153]. Both dFMRP and DmGluRA regulate iGluR subclass abundance within single postsynaptic domains; however, the regulative mechanisms appear convergent rather than linear [145]. Null *dfmr1* mutants upregulate A-class and downregulate B-class iGluRs, whereas *DmGluRA* mutants increase both receptor classes in parallel. The *dfmr1*;*DmGluRA* double-null mutant displays the additive consequences of these two changes [145], suggesting convergent regulation of iGluR trafficking. Notably, other recent work at the *Drosophila* NMJ has revealed that immature postsynaptic densities maintain a higher ratio of GluRIIA to GluRIIB subunits, becoming more balanced with synaptic maturation correlated with the arrival of the active zone protein Bruchpilot [154]. Further, expressing tetanus toxin light chain to reduce presynaptic glutamate release increased GluRIIA abundance but decreased GluRIIB incorporation. These findings suggest that *dfmr1* nulls may be arrested in a functionally immature postsynaptic state.

### Understanding Key FMRP/mGluR Issues in *Drosophila*

Together, these studies strengthen the connection between DmGluRA signaling and dFMRP function and further implicate enhanced mGluR signaling as one of the causative factors leading to impaired cognitive function and neuroanatomical changes in FraX. Though enticing, conclusions about the "mGluR theory" in *Drosophila* are complicated by several factors. Contrary to vertebrate mGluR1/5 group I receptors that drive $G\alpha_q$-mediated phospholipase C activation generating diacylglycerol and inositol 1,4,5-triphosphate [120], DmGluRA is most similar to group II/III mGluRs [143, 148]. These receptors display $G\alpha_{i/o}$-coupled signaling, which mediates adenylyl cyclase inhibition of cyclic adenosine monophosphate (cAMP) formation [120]. Nevertheless, as the sole *Drosophila* mGluR, DmGluRA likely mediates all known mGluR signaling events, such as ERK activation important in mGluR-dependent LTD [155] and thus may function via additional conserved $G\alpha_q$-dependent mechanisms. Supporting this idea is *Drosophila* work with the mammalian mGluR5-specific antagonist, MPEP, a highly lipophilic and noncompetitive inhibitor that interacts with the receptor transmembrane domain [156, 157]. In *Drosophila*, the predicted secondary structure constituting the MPEP binding pocket is conserved in DmGluRA, and a genomic database BLAST search for this sequence reveals DmGluRA as the only predicted target [143]. Importantly, MPEP appears to quite readily antagonize DmGluRA signaling to ameliorate *dfmr1* mutant phenotypes, paralleling results obtained with *DmGluRA* genetic mutants [32, 143, 145, 146]. In addition, however, competitive antagonists acting on the glutamate-binding pocket of mammalian mGluR groups II/III [158], including (RS)-α-methyl-4-phosphonophenylglycine, (RS)-α-methyl-4-tetrazolylphenylglycine (MTPG), and LY341495, also appear to counteract *dfmr1* phenotypes [143], although they have not been used extensively. With these caveats in mind, attenuation of DmGluRA signaling needs to be carefully examined as nonpredicted coincident pathways may be influenced in the attempt to ameliorate particular neurological phenotypes.

With the *dfmr1* mutation established as an appropriate, meaningful, and powerful genetic model system to explore the molecular underpinnings of FraX, full exploitation of its utility in dissecting associated behavioral phenotypes is emerging. In conjunction with mammalian *Fmr1* studies, significant progress has been made recently in the *Drosophila* FraX model to define the neuronal circuits and molecular genetic pathways contributing to the clinically relevant alterations in circadian rhythms and deficits in learning and memory.

## Biological Rhythm Impairment in FraX

### Circadian Activity Abnormalities in FraX

FraX patients display altered melatonin levels and disrupted activity patterns characterized by hyperactivity and disordered sleep [46, 47]. Similarly, *dfmr1*-null mutants display altered activity profiles, disrupted sleep, and striking circadian rhythm phenotypes, hallmarked by arrhythmicity and erratic locomotion [137, 139, 141, 142]. In normal animals, coordinated clock circuit function programs 12–14 h of daytime activity, peaking at dawn and dusk, and 10–12 h of inactivity at night [159, 160]. This patterned activity profile is maintained even in total darkness, indicating a sustained and innate circadian rhythmicity [161, 162].

Morales et al. [139] reported that *dfmr1*-null mutants engage in lower average levels of activity. In contrast, Dockendorff et al. [137] reported that average activity

levels were comparable between control and mutant animals but that *dfmr1* nulls occasionally exhibit bouts of extreme hyperactivity. Similarly, hyperactivity has been reported in some analyses of *Fmr1*-null mice [136, 163]. These observations may resemble clinical findings of copresentation of FraX and attention deficit hyperactivity disorder [164, 165]. More robustly, several studies have shown that *dfmr1* nulls lack bimodal crepuscular patterns of rest and activity [137, 139, 141, 142]. Null mutants remain light entrainable, but the vast majority (>85%) displays weak and erratic circadian patterns or complete arrhythmicity when challenged with constant darkness. Further, *dfmr1* overexpression induces a lengthening of the measurable period associated with the rescue of patterned activity profiles, providing further support for a specific requirement for dFMRP in circadian regulation [137, 141]. The contribution of the RNA-binding KH domains to this phenotype was demonstrated by mutational substitution of critical conserved isoleucine residues (I244N and I307N); failure to retain rhythmicity resulted, albeit to a lesser degree than in full *dfmr1*-null mutations [166]. This finding indicates partial loss of function upon disruption of RNA-binding domains and, furthermore, that the RNA-binding properties of each domain are specific, as the unaltered RGG box was noncompensatory. Perhaps surprisingly, an independent study demonstrated that, despite anticipated attenuation of these defects with decreased mGluR signaling, treatment with the noncompetitive mGluR antagonist MPEP failed to resolve the circadian behavioral impairments in *dfmr1* nulls [143].

As a result of these *Drosophila* FraX model studies, similar assays of circadian behavioral rhythms have been pursued in mice [167]. The role of mammalian FMRP is complicated by the presence of the three-member gene family (*FMR1*, *FXR1*, and *FXR2*), which encodes proteins that share >60% amino acid identity and conservation of functional domain organization [168, 169]. The presence of homotypic and heterotypic complexes between these three proteins may confound experimental interpretations if any family member is able to partially compensate for loss of the other. Thus, a combinatorial approach has recently been employed to simultaneously remove two family members. Mice lacking either FMRP or FXR2P alone maintain fairly normal rhythmicity, albeit with an associated shortening of free-run periodicity when released into constant darkness [167]. However, the *Fmr1/Fxr2* double-null animals are entirely arrhythmic and furthermore apparently lack all entrainment capacity [167]. Thus, circadian regulation of behavior is critically dependent on FMRP in both the *Drosophila* and mouse FraX models.

## Circadian Clock Circuit Abnormalities in *FMR1* Mutants

The *Drosophila* circadian clock is driven by cyclic transcription feedback loops that maintain regular rhythms [161, 162, 170, 171]: two basic helix–loop–helix transcription factors, clock and cycle, heterodimerize and activate the transcription of period (*per*) and timeless (*tim*). PER and TIM proteins shuttle to the nucleus and autorepress by inhibiting the action of CLK and CYC. Despite severely disrupted circadian activity, *dfmr1* nulls exhibit normal oscillations of PER and TIM at both mRNA and protein levels [137, 139, 141], suggesting that the clock proper functions independently of dFMRP. One subtle PER/TIM-associated clock irregularity does manifest under conditions of constant darkness, i.e., the electrophoretic shifts in PER and TIM due to phosphorylation state are moderately out of phase [141]. While compelling, further examination of the PER/TIM oscillations under constant darkness, at the individual clock neuron level [137] and over extended time periods [141], would extend these findings and allow formal comment on the maintenance or fatigue of resident pacemaker functions. Similarly, despite altered behavioral rhythmicity, the mammalian central circadian pacemaker in the suprachiasmatic nucleus (SCN) displays no overt clock defect associated with loss of FMRP, FXR2P, or both in *Fmr1*/*Fxr2* double-mutant mice [167]. Rhythmic expression patterns of the clock gene mRNAs *Per1*, *Per2*, *Bmal1*, and *Cry1*, as well as SCN electrical output activity, appear normal. Surprisingly, however, clock gene expression in the liver was altered by the loss of FMRP, FXR2P, or both, indicating a peripheral clock impairment [167]. While these results appear contradictory, assessment of *mPer1/2* promoter-linked transcriptional activity using a luciferase reporter indicated BMAL1-NPAS, but not BMAL1-CLOCK, liver and brain transcription factor combinations, respectively, to be activated by FXR2P alone or in conjunction with FMRP [167]. Thus, FMRP and FXR2P influence the molecular clock feedback loop mediated by the liver-specific BMAL1-NPAS2 rather than the central BMAL1-CLOCK pathway.

Despite this complication, in the brain, it appears that the circadian clock itself operates normally in the complete absence of FMRP. Thus, presumably the neural outputs mediating behavioral rhythmicity are perturbed in the *Fmr1*-null mutants. Examining this hypothesis in *Drosophila* has resulted in variable findings. A known clock output, the neuropeptide pigment-dispersing factor (PDF) [172, 173], cycles regularly within the termini of specific clock neurons [137]. However, the clock-controlled gene, cAMP response element binding protein transcription factor, is disturbed by *dfmr1* mutation [137]. Thus, it is of particular interest that dFMRP and another known clock output component and RNA-binding protein, LARK, were recently reported to function together in the regulation of circadian behavior in *Drosophila* [174]. dFMRP and LARK complex *in vivo*, with dFMRP levels being coincidently depressed upon LARK reduction in the whole animal, suggesting that LARK may influence dFMRP translation or stability.

However, knockdown of LARK within specific clock neurons results in elevated dFMRP, and LARK overexpression likewise mediates dFMRP reduction [174]. These differential relationships suggest that the two RNA-binding proteins may serve to function cooperatively, or in opposition, to regulate circadian component translational regulation in a context-dependent manner.

The *Drosophila* circadian clock circuitry is particularly well described, with the clock neurons being divided into two main categories, dorsal (DN) and lateral (LN) neurons (Fig. 2). These are further subdivided into the following categories [162, 170, 171, 175]: DN1, DN2, DN3, dorso-LN ($LN_d$), and the large and small ventro-LN ($lLN_v$ and $sLN_v$, respectively, Fig. 3a), which express the neuropeptide PDF. Other neurons involved in circadian rhythms include lateral posterior neurons (LPN), which display oscillations of PER and TIM proteins but have not been shown to have pacemaker functions, the Hofbauer-Buchner eyelet, which relays photic entrainment signals to the $sLN_v$, the crustacean cardioactive peptide-expressing neurons (CCAP), which control eclosion circadian behaviors, prothoracic gland innervating neurosecretory neurons of the lateral protocerebrum (PG-LP) and, lastly, neurons of the pars intercerebralis in the dorsal protocerebrum (PI-3 neurons), which receive projections from the $LN_v$s, $LN_d$, and DNs, and thus likely serve as pacemaker target cells (Figs. 2 and 3a) [162, 170, 171]. Initial analyses of this circuitry in *dfmr1* mutants have revealed striking abnormalities in the architecture of the $LN_v$s (Fig. 3b), a key subset of clock neurons that possesses a circadian pacemaker sufficient to modulate morning activity peaks in light–dark cycles and drive rhythmicity in constant darkness [176–178]. Null *dfmr1* $LN_v$s neurons display overgrowth, overextension, and mistargeting beyond the normal dorso-medial branching and defasiculation point in the protocerebrum, posterior optic tract splitting, and an increased frequency of aberrant collateral branching (Fig. 3b) [89, 137, 139]. Conversely, dFMRP overexpression results in a dramatic collapse of the $LN_v$s dorsal axonal arbor [89]. These apparent pathfinding and axon guidance defects suggest that *dfmr1* mutant $LN_v$s may fail to recognize, or respond to, local instructive cues required to stop neurite extension and establish appropriate synaptic contacts with target neurons. Mechanistically, mutational analysis shows that the dFMRP N-terminal region interacts with the small GTPase Rac1 interactor CYFIP [93, 179], cooperatively regulating axonal projection and synaptic development in these $LN_v$s clock neurons [147]. Misregulation of the actin-binding protein Chickadee is also directly linked to architectural defects in the circadian $LN_v$s, as decreasing Chickadee levels suppresses the *dfmr1*-null phenotype [89]. This clock circuit will be a particularly fruitful context to pursue parallel investigations into the role of FMRP in neural circuit formation and a directly mediated output behavior.

## Cognitive Dysfunction in FraX

### Associative Learning and Memory Deficits in FraX

A striking behavioral consequence in FraX patients is the disruption of working memory (WM), i.e., the temporary

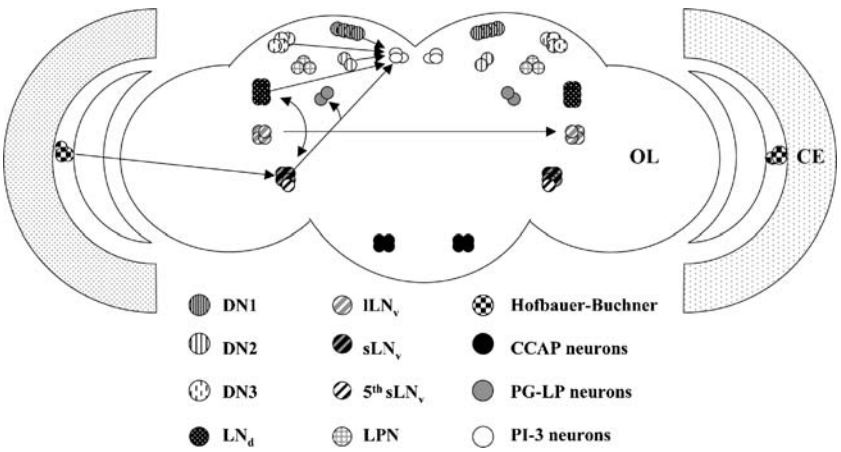


**Fig. 2** *Drosophila* clock circuitry. The *Drosophila* circadian clock circuitry is diagrammed with neuronal class positioning as indicated. Connectivity patterns are highlighted unilaterally with *black arrows* for clarity. Photic information is relayed from the compound eye (*CE*) via the Hofbauer-Buchner eyelet projecting to the central brain through the optic lobe (*OL*). Therein, the lateral neurons (*LN*) [dorso-LN ($LN_d$), the large and small ventro-LN ($lLN_v$ and $sLN_v$, respectively), and the fifth non-pigment-dispersing factor expressing $sLN_v$] work in concert with the dorsal neurons (*DN1*, *DN2*, and *DN3*) to maintain rhythmicity. Other clock-associated neurons include the lateral posterior neurons (*LPN*), the crustacean cardioactive-peptide-expressing neurons (*CCAP*), and the prothoracic gland neurons (*PG-LP*). Lastly, the apparent target neurons of the pars intercerebralis in the dorsal protocerebrum (*PI-3 neurons*) receive input from several clock neuron classes. Modeled with permission after [170]

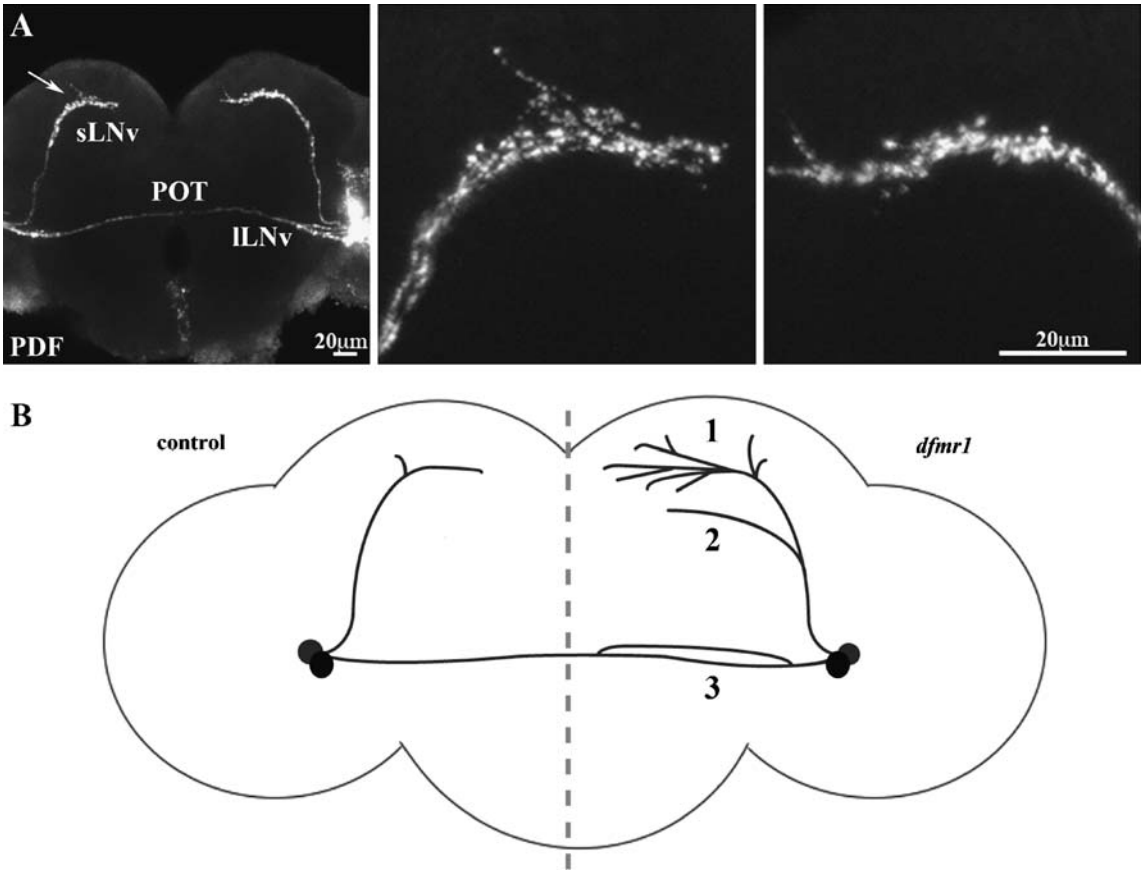


**Fig. 3** Null *dfmr1* abnormalities in lateral clock neurons. **a** Representative images of adult *Drosophila* central brain regions immunohistochemically labeled for pigment-dispersing factor (*PDF*), a neuropeptide expressed only in ventrolateral neurons ($LN_v$). *Arrow* indicates the dorsal horn bifurcation point of the small ventrolateral neurons ($sLN_v$). The large ventrolateral neurons ($lLN_v$) mediate interhemisphere connectivity via the posterior optic tract (*POT*). Higher magnification views of the left and right $sLN_v$ terminal axonal projections are shown. **b** Cartoon summarizing the primary $LN_v$ axonal phenotypes observed in null *dfmr1* animals. The complex $lLN_v$ dendritic and terminal axonal elaborations within each ipsilateral and contralateral optic lobe, respectively, have been omitted for simplicity. Wild-type $LN_v$s (left hemisphere) maintain stereotyped and contained projection pathway patterns. Null *dfmr1* $LN_v$s (right hemisphere) display (1) overextension and mistargeting beyond their normal dorsomedial branching and defasiculation point, (2) an increased frequency of aberrant collateral branching, and (3) POT splitting [89, 137, 139, 147]

storage, manipulation, and retrieval of learned information. In particular, WM tasks that require more substantial attentional focus, e.g., two-back tasks, show the most significant deficits in FraX patients [42–45]. Functional neuroimaging in these patients indicates an inability to modulate activation in the prefrontal and parietal cortex [42], regions thought to be key in WM [180, 181]. This impairment may be a key component of the cognitive challenge facing FraX patients.

Mouse FraX models have shown variable results in attempts to identify memory defects. Hippocampal-dependent paradigms (e.g., water and radial maze assays) have revealed only subtle performance differences, including substandard spatial learning and decreases in escape latency and path length [136, 163, 182–186]. The appearance of these defects has been inconsistent and often dependent upon genetic background [183, 185]. Fear-conditioning experiments have yielded similarly inconsistent results [183–185], although analysis of the *Fmr1*/*Fxr2* double KO demonstrated significantly more depressed behavioral modification than either single mutant [187]. Nonhippocampal learning and memory assays appear to provide more robust and readily identifiable phenotypes. Cerebellar eyelid conditioning is significantly reduced upon removal of FMRP, both in the global *Fmr1*-null and Purkinje-cell-specific *L7-Fmr1* deletion [188]. Importantly, recent findings show that associated measures of altered prepulse startle inhibition are largely rescued with mGluR inhibition via MPEP treatment [124]. In trace fear memory, involving the anterior cingulate cortex and requiring elevated attentional dedication as the unconditioned stimulus is separated temporally from the conditioned stimulus, *Fmr1*-null animals display deficits in both the acquisition and retention phases [189]. These defects are partially alleviated upon coincident disruption of the p21-activated kinase effector of Rac1 [190]. Lastly, *Fmr1* KO animals are less

responsive in aversively motivated and striatally mediated lever press escape/avoidance tasks [191].

Until recently, learning and memory assays in the *Drosophila* FraX model have been based solely on experience-dependent modification of courtship behavior [137, 143]. In these analyses, a naïve male is paired with a previously mated female, with courtship behavior scored. In normal animals, courting attempts decline as receptivity is diminished. Typically, the subsequent presentation of a virgin female fails to promote courtship, which remains depressed in the male for several hours as a result of memory acquisition [192–194]. In this paradigm, null *dfmr1* mutants are capable of normal learning, as immediate behavioral adaptation during training is indistinguishable from wild type [143]. However, persistence of the associated behavioral modification is absent, during windows examining both immediate recall and short-term memory (STM), indicating that mutants fail to translate this learning into accessible consolidated memory. Notably, these memory defects can be corrected with the mGluR antagonist MPEP or a more general lithium treatment [137, 143], implicating that the decrement of mGluR-related effects via differential pathways may prove to be therapeutic.

The best-characterized associative learning and memory behavior in *Drosophila* is a cue-based olfactory paradigm [195–197], which has just recently been rigorously applied to the *Drosophila* FraX model [32]. This Pavlovian assay employs a learning phase, delivering two matched repulsive odors (e.g., octanol and methylcyclohexanol) sequentially, with one being paired with electric shock and the other not, and a memory phase, examining the retention of conditioned training by requiring that a spatially segregated odorant choice be made. Learning is examined immediately after training and is known to be protein synthesis independent [195]. Memory is tested at progressive periods through STM and intermediate amnesia-resistant memory (ARM), both of which are also protein synthesis independent, and long-term memory (LTM), which is known to absolutely depend on *de novo* protein synthesis [195].

Utilizing this paradigm in the *Drosophila* FraX model, *dfmr1*-null animals display moderately impaired immediate associative learning [32]. Since there is no hypothesized role for RNA regulation during initial learning, this defect may reflect a developmental impairment of the neural circuitry underlying the innate ability to learn. The learning disability is quite mild, and the performance index is sufficiently high to examine memory retention. Massed training to produce STM and ARM is intact in *dfmr1*-null animals, showing that there is no requirement for dFMRP in these protein-synthesis-independent events [32]. However, tests of 24-h protein-synthesis-dependent LTM after spaced training reveal that *dfmr1* nulls exhibit a severely impaired memory performance index, showing that dFMRP plays a vital role in memory consolidation. A similar recent study employed olfactory discrimination using a "simultaneous-cue, two-odor, forced-choice paradigm" as a means of examining olfactory learning and memory in *Fmr1*-null mice [198]. Interestingly, an analogous learning impairment was described with the mutant mice requiring significantly more time to ascertain criterion performance for any given discrimination problem. In contrast, however, once trained, LTM was intact at both 2 days and 4 weeks [198]. In the *Drosophila* model, strong upregulation of dFMRP in the wild-type brain was induced after spaced, but not massed, training [32]. Note that dFMRP upregulation occurs only under conditions that induce protein-synthesis-dependent memory consolidation. The memory defect also shows tight spatial specificity, as dFMRP knockdown via *dfmr1* RNAi expressed solely within the MB is sufficient to impair LTM [32]. Lastly, this LTM defect is rescued in *dfmr1* mutants by expressing a genomic *dfmr1* transgene and ameliorated by either protein synthesis inhibition (i.e., puromycin, cycloheximide) or mGluR antagonism (i.e., MPEP) [32], providing further evidence of dFMRP function as a translational regulator downstream of DmGluRA signaling.

dFMRP interacts with other known memory-associated components. Staufen is a double-stranded RNA-binding protein that colocalizes with FMRP in dendritically trafficked RNA granules [22, 199–201] and has been implicated in LTM [202]. In *Drosophila*, animals heterozygous for either dFMRP or Staufen maintain normal memory profiles [32]. However, double heterozygous mutants, concurrently reducing the levels of both dFMRP and Staufen, display reduced 24-h protein-synthesis-dependent LTM after spaced training. In addition, dFMRP has been identified as a RISC complex component interacting with Argonaute1 (Ago1), which facilitates processing in the mediation of translational control by miRNAs [23]. Animals heterozygous for either dFMRP or Ago1 maintain normal memory profiles but, as with Staufen, double heterozygotes with reduced dFMRP and Ago1 levels are deficient in post-spaced-training LTM [32]. The role of RISC in *Drosophila* memory was initially elucidated upon mRNA transport and synaptic protein synthesis studies of the dFMRP target CaMKII. Upon neural stimulation and/or olfactory training, *CaMKII* mRNA is upregulated and dendritically trafficked. RISC then regulates CaMKII expression posttranscriptionally, likely utilizing the miR-280 binding site within the *CaMKII* 3′UTR [203]. These results suggest cooperativity among several classes of RNA-binding proteins in the appropriate regulation of proteins required in the generation or maintenance of LTM.

## Learning and Memory Circuit Abnormalities in *FMR1* Mutants

Alterations in neuronal structure and connectivity may be associated with the cognitive impairments in FraX. FraX patients present elevated densities of long, thin, and immature-looking cortical dendritic spines, indicating inappropriate development and/or failure of pruning and synapse elimination [55, 56], with similar findings reported in *Fmr1*-null mice [136, 204–206]. In the mutant mouse hippocampus, decreased intrapyramidal and infrapyramidal mossy fiber terminal fields [163], altered zinc-rich Timm granule-staining terminals within the CA3 and the inner molecular layer [207], and immature dendritic spine profiles in the CA1 pyramidal region [208] are observed. Organotypic slice cultures reveal that *Fmr1* KO neurons possess more morphologically defined synapses than their wild-type counterparts [209]. *In vitro* studies present a more complicated picture. Mouse mutant hippocampal neurons grown in culture for 3 weeks display shortened dendrites and fewer dendritic spines as compared to wild type, with fewer synaptophysin-reactive puncta and depressed excitatory synaptic currents [210]. Another report examining neurons after 16 days *in vitro* (DIV) demonstrated that *Fmr1*-null neurons present excessive filopodia and longer spines, with a reduction in the number of projections that are adjacent to presynaptic specializations demarcated by synapsin immunoreactivity [211]. A very recent study indicated that, at 21 DIV, *Fmr1*-null neurons maintain normal dendritic branching patterns and spine densities but display a disproportionate number of filopodial extensions as compared to controls [124]. Further, this filopodial excess was abrogated upon acute culture treatment with the mGluR5 antagonists MPEP and fenobam [124].

Of particular relevance to learning and memory centers, and in light of impairments underpinning the "mGluR, and more generalized Gq, theory of FraX" [40, 133], are the functional plasticity perturbations in cortical long-term potentiation (LTP) [212–214] and hippocampal LTD [38, 39, 133]. Within the cortex of *Fmr1* mutant mice, reduced LTP is associated with a concomitant decrease in AMPAR subunit GluR1 trafficking [212, 215]. Additionally, the typical LTP attenuation provided by the general mGluR antagonist alpha-methyl-4-carboxyphenylglycine (MCPG) and the selective mGluR5 antagonist MPEP did not further depress this reduced LTP, suggesting that this form of synaptic plasticity is fundamentally absent [214]. However, findings examining spike-timing-dependent LTP (tLTP) revealed that neocortical plasticity is not entirely absent, but its induction threshold is elevated in the absence of FMRP [213]. This defect is associated with impaired calcium signaling, characterized by dendritic failure to exhibit calcium transients and absence of L-type calcium channel activity. Of particular interest, increasing neuronal activity restored tLTP, suggesting alternative intact plasticity mechanisms [213]. Recent data implicate such an alternative with the enhancement of signaling via the small GTPase Ras/PI3K/protein kinase B (PKB,Akt) pathway mediating the synaptic delivery of GluR1-containing AMPAR and restoration of normal LTP in *Fmr1*-null mice [215]. As previously discussed, studies of hippocampal mGluR-initiated protein-synthesis-dependent LTD reveal an elevation of this state in the *Fmr1* mutant animals upon induction via both PP-LFS and agonist application (i.e., DHPG), while mGluR- and protein-synthesis-independent NMDAR-LTD remains normal [38, 39]. Further, the *Fmr1*-associated elevation in LTD and corollary decrease in AMPAR membrane expression are uncoupled from protein synthesis, as both persist upon introduction of anisomycin [38]. Similarly, silencing RNA directed against *Fmr1* in cultured hippocampal neurons demonstrated excessive AMPAR internalization likely serving as the causative mGluR-triggered event in the enhanced LTD FraX condition [105].

The primary learning and memory center characterized within the *Drosophila* brain is the MB (Figs. 4 and 5a) [216, 217]. The cell bodies of the ~2,500 individual MB Kenyon cells are located on the dorsal posterior aspect of the brain in each hemisphere. The dendritic field is confined to the region known as the calyx, wherein olfactory receptor neuron-coupled input from the projection neurons is received. From the calyx, the axons project together as one fasiculated unit, the peduncle, beyond which axonal bifurcation and projection patterns then place the MB neurons into three distinct classes: αβ, α'β', and γ [218, 219]. In *dfmr1*-null animals, both axonal and dendritic structural changes have been reported. Initial studies showed no gross-level MB defects [137], while it was later reported that β-lobe axons fail to terminate short of the midline, resulting in inappropriate extension into the contralateral MB lobe (Fig. 5b) [138]. Much higher resolution single-cell-level analyses have been done using the mosaic analysis with repressible cell marker system [220, 221], a genetic clonal approach to study cell-autonomous dFMRP function. Null *dfmr1* MB neurons display structural overelaboration, including excess process initiation resulting in supernumerary processes, overgrowth and branching of axonal processes, and increased dendritic field complexity (Fig. 5b) [144]. Conversely, dFMRP overexpression results in neuronal oversimplification and underbranching, showing that dFMRP levels can bidirectionally regulate growth. Very recently, it was shown that dFMRP negatively regulates MB axonal elongation during late brain development, consistent with the above results, while temporally shifting an activity-dependent phase of process pruning upon initial use of the circuit [15].

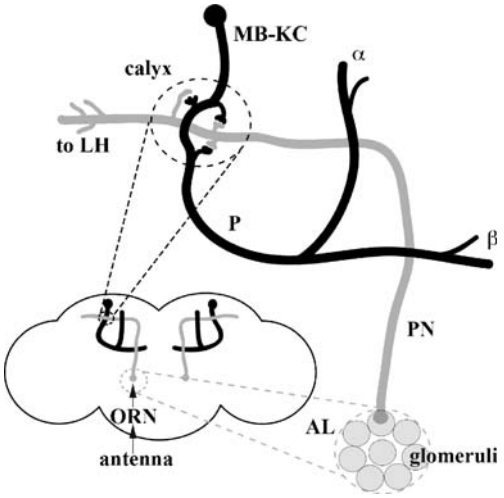


**Fig. 4** *Drosophila* olfactory learning and memory circuitry. *Drosophila* olfactory learning and memory involves a circuit that mediates the transduction of information initially encountered at the antennae via the olfactory receptor neurons (*ORNs*) to the projection neurons (*PNs*) organized within the glomeruli of the antennal lobe (*AL*). The PNs then extend to both the mushroom body Kenyon cells (MB-KC) and further to the lateral horn (*LH*). The synaptic field of the PN and MB-KC is spatially restricted to the region known as the calyx. Upon projection from the calyx and peduncle region (*P*), MB-KC bifurcation and projection patterns establish the MB lobes themselves ($\alpha$ and $\beta$ indicated)

Consistent with the "mGluR Theory," mGluR antagonists (e.g., MPEP, MTPG, LY341495) restore MB structure towards normal, both at the level of gross MB anatomy and, for MPEP, at the level of individual MB neuron architecture [143, 146]. Taken together, the results from both *Drosophila* and mouse FraX models indicate that FMRP plays a conserved role in achieving and/or maintaining appropriate architecture in neuron classes involved in learning and memory.

## The Spatiotemporal View of FraX

Complications in approaching FraX pathophysiology across all disciplines arise from the basic questions of when and where FMRP is required. Despite clear symptomatic clinical presentations, it remains to be decisively demonstrated whether FraX is a neurologically "developmental disease," reflecting an early transient requirement for FMRP, a "plasticity disease," reflecting a maintained requirement for FMRP at maturity, or some combination giving rise to different FraX symptoms. Further, as FraX is largely characterized by discrete neuronal defects, the spatial specificity associated with the mechanistic cellular actions and functionality of FMRP warrants close consideration. Collectively, these spatiotemporal examinations may well prove critical to the design and implementation of effective therapeutic strategies. In particular, the temporal action of FMRP is key to understanding FraX onset and progression. FMRP may play a definitive role during development, fundamental to establishing appropriate networks of neuronal connectivity, with early expression likely predisposing normal functionality at later time points. However, this idea is not mutually exclusive of a biphasic model of FMRP also playing an acute role in synaptic plasticity in the mature brain. In fact, a plasticity function may be the most relevant to alleviating cognitive dysfunction after clinical diagnosis, and a number of results suggest that FMRP-mediated effects are not solely confined to one temporal window.

### Temporal Requirements for FMRP: Roles in Development Versus Maturity

In FraX patients, some symptoms are persistent whereas others are age dependent. In the latter class, for example, seizures affect many FraX children, remitting in most patients by the end of adolescence [48–51]. Similarly, the mouse FraX model displays a number of defects that appear transiently during development. The *Fmr1*-null mice are extremely susceptible to audiogenic seizures [123, 222–224], typically showing fully manifest heightened sensitivity by weeks 2–3 of postnatal life [123, 223]. Some reports indicate mutant animals subsequently completely lacking seizures by 4–7 weeks, depending on genetic background [123]. Similarly, mutant mice possess abnormally increased dendritic spine length and density in layer V of the somatosensory barrel cortex in early development (postnatal week 1), corresponding to the typical period of robust cortical synaptogenesis, but these defects are much attenuated by week 2 and entirely undetectable by week 4 [225, 226]. Further investigation suggests the later reappearance of this condition in adult mice (approximately week 10–11) [225], indicating a very complex temporal dynamic. Similarly, a recent study investigating barrel cortex circuitry development in *Fmr1*-null mice demonstrated transient defects in the ascending projection connecting layers III and IV [227]. At postnatal week 2, the excitatory circuit was aberrant with reduced transmission strength and associated axonal arbor disorganization with the projection showing no functional experience-dependent plasticity, i.e., depression upon sensory deprivation by whisker trimming [227]. These defects appear fully resolved by postnatal weeks 3–4, demonstrating similar transient defects in structure and function in the barrel cortex. Unfortunately, no later time points were examined to address the potential resurrection of the functional phenotype. In contrast, acquisition of the startle response in auditory development is initially FMRP independent, with both control and *Fmr1* mutant animals displaying normal response onset and

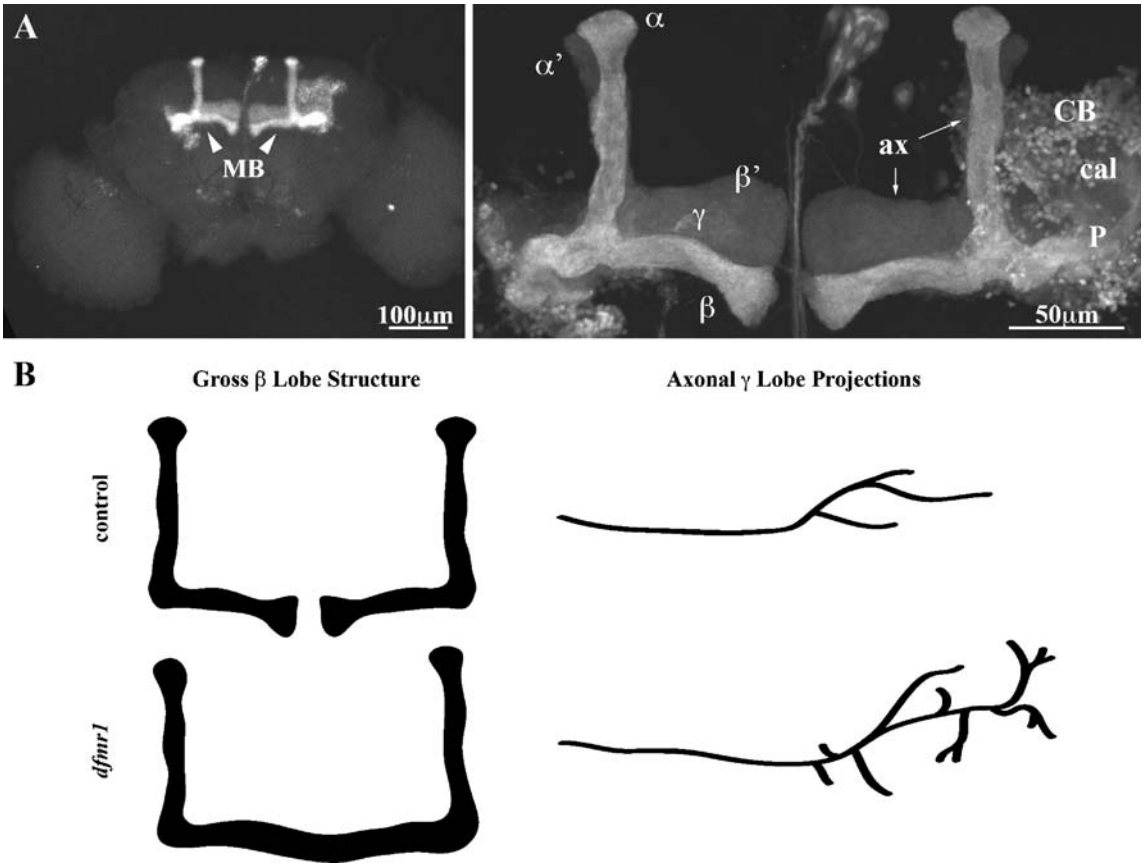


**Fig. 5** Null *dfmr1* abnormalities in mushroom body neurons. The *Drosophila* mushroom body (*MB*) illuminated via the combination of a MB-specific GAL4 genetic element driving the expression of a UAS-GFP transgene. Note the bilateral symmetry of these structures. Anatomically, beyond the cell bodies (*CB*), individual MB Kenyon cells put forth their dendritic specializations, which, as stated, are concentrated in the calyx (*cal*). The axons then project together, as the fasiculated peduncle (*P*), before specific bifurcation and differential projection patterns then determine the MB neuron's residence within a given axonal lobe (*ax*): $\alpha\beta$, $\alpha'\beta'$, and $\gamma$. **b** Cartoon summary of two well-characterized MB phenotypes associated with *dfmr1* insufficiency. In *dfmr1*-null mutants, β-lobe axons fail to terminate approaching the midline, resulting in inappropriate extension into the contralateral MB lobe and β-lobe fusion [138, 143]. At single-cell resolution, null *dfmr1* MB neurons display structural overelaboration, including enhanced process initiation resulting in excessive overgrowth and branching of axonal processes (depicted) and increased dendritic field complexity (not shown) [15, 144, 146]

amplitude; however, progression is prematurely arrested in the mutant beyond postnatal week 3 yielding a diminished startle response at maturity [228]. Although most murine phenotypes show transient manifestation in very young animals, an exception has been reported in another brain region, the anterior piriform cortex. In *Fmr1*-null mice, theta-burst stimulation-induced LTP was reported impaired only in animals at least 6 months in age and became more pronounced over time [229].

Recent work in the *Drosophila* FraX model has similarly highlighted the transient temporal roles of dFMRP in neuronal development. Null *dfmr1* MB neurons display both axonal and dendritic overelaboration, with defects in the architecture of synaptic connections [144]. To identify potentially critical periods for the appearance of these defects, systematic determination of developmental dFMRP expression patterns and functionality in relation to modulating a target mRNA was undertaken [15]. First, both *dfmr1* transcript abundance and dFMRP protein levels were elevated during pupal development with evident decline upon eclosion to maturity. Interestingly, in the aging adult, dFMRP protein levels remain minimal, apparently uncoupling from mRNA levels which themselves rebound establishing a secondary peak [15]. This temporal pattern precisely parallels a recent report detailing the expression of *Fmr1* and FMRP in the brains of aging female mice [230] and reflects the often reported trend of an early peak of FMRP expression followed by progressive age-dependent protein level diminution [17, 231–233]. Accordingly then, MB neuron structural defects were determined to first appear only very late in pupal brain development, with the appearance of abnormally long primary axonal branches and associated excessive short synaptic processes. In wild type, many of these short processes are pruned away in the young animal upon initial use, presumably eliminating excess supernumerary synapses [15]. In contrast, *dfmr1*-

null neurons completely fail to manifest activity-dependent pruning, leading to the retention of excess synapses and the overelaborated mutant morphologies. However, in adult animals, compensatory and moderately excessive pruning restores normal architecture at maturity. Consistent with such an activity-dependent mechanism, dFMRP expression is positively regulated by sensory input activity, while expression of known dFMRP targets (e.g., Chickadee/Profilin) is negatively regulated by activity [15]. Moreover, sensory deprivation alone resulted in the failure of this FMRP-mediated pruning in control animals and exacerbated the defect in *dfmr1* mutants. Transgenic introduction of an exogenous channel to elevate neuronal activity resulted in increased activity-dependent pruning in wild type, but not in *dfmr1* nulls, directly demonstrating that activity-dependent pruning requires dFMRP [15]. Thus, in both mouse and *Drosophila* FraX models, there is a transient window of FMRP requirement that corresponds to the initial period of use that drives activity-dependent refinement of neural circuits.

In *Drosophila*, developmental-stage-targeted transgenic rescue studies and timed pharmacological interventions have also been informative in defining the temporal periods of dFMRP function. In assays of *Drosophila* courtship behavior, *dfmr1*-null animals were treated with mGluR antagonists during larval development, as adults, or both [143]. While treatment at adulthood ameliorated behavioral defects, efficacy was improved when the agents were presented during development. Similarly, a recent study of the *Drosophila* neuromuscular synapse revealed temporally sensitive FMRP requirements [88]. Null *dfmr1* synapses display excessive branching, overgrowth, and increased synaptic bouton numbers [83, 88, 146]. A strategy was employed to reintroduce neuronal dFMRP in the otherwise *dfmr1* null via a hormone-responsive genetic element using the Gene-Switch System [234, 235]. Constitutive neuronal dFMRP expression in the *dfmr1* nulls resulted in rescued synapses indistinguishable from wild type [88]. Importantly, early dFMRP induction paradigms, with expression only during the earliest period of post-embryonic-development, effected nearly complete rescue of synaptic architectural defects when assessed at larval maturity [88]. In contrast, acute dFMRP induction at maturity enabled only limited resolution of the mutant defects. These results suggest that dFMRP plays an important role in early development that underlies persistent defects in synaptic architecture and behavioral outputs.

## Spatial Requirements for FMRP: Presynaptic Versus Postsynaptic Roles

Loss of FMRP function results in substantial morphological disruption across a variety of neuronal classes [15, 83, 88, 89, 92, 137–139, 144–147], with mammalian *Fmr1* structural studies largely focusing on the specific alterations to the cortical and hippocampal dendritic spines [124, 205, 206, 208, 210, 211, 226, 236]. Long-standing evidence indicates that dendritic spines are dynamic structures that can be formed, modified, or eliminated in an activity-dependent manner [237–239]. Incoming axonal projections converge upon dendritic spines, which serve as posts of signal input integration, wherein local protein synthesis likely promotes synapse-specific plasticity [240, 241]. In general, the dendritic spine phenotypes that present in the absence of FMRP are discussed as entirely postsynaptic defects. However, presynaptic and postsynaptic specializations are requisite for *de facto* functional synapse formation [242]. Therefore, FMRP could be required presynaptically, postsynaptically, or transsynaptically to generate the observed defects.

Mammalian studies provide evidence to bolster each of these hypotheses. FMRP is present in both axonal growth cones and dendritic spines [22, 81, 82]. Growth cones lacking FMRP show excessive filopodial formation and reduced motility, suggesting potentially compromised synaptogenic potential [211]. In support of this, a mosaic mouse model that mirrors the X-inactivation present in female FraX patients indicates that presynaptic *Fmr1* genotype influences synaptic connectivity in CA3 pyramidal neurons of organotypic slice preparations [243]. Examination of native synaptic partners by whole-cell recording in this system revealed that presynaptic *Fmr1*-null neurons are less likely to form functional synapses, whereas the postsynaptic *Fmr1* status played no role [243]. Contrary to these findings, a study using transfection of cultured primary neurons indicated that acute (3–7 days) postsynaptic FMRP expression induces synapse loss in these cells [209]. Interestingly, the KH2 RNA-binding domain, and not the RGG box, was required to mediate these synaptic effects. However, the interpretation of these results is complicated by the lack of FMRP level normalization and the likelihood that transfection yielded FMRP overexpression phenotypes.

A recent *Drosophila* study implicated differential and separable roles for dFMRP in modulating synapse structure and function in presynaptic and postsynaptic cells, respectively [88]. By examining the neuromuscular synapse, with its readily distinguishable presynaptic and postsynaptic cells, it is possible to tease apart requirements with muscle- and neural-specific transgenic drivers [244]. Targeted presynaptic reintroduction of dFMRP in *dfmr1*-null animals restored totally normal synapse morphology, bouton deposition, and cytoskeletal organization [88]. Thus, these architectural features are driven by dFMRP requirement in the presynaptic compartment. Functional neurotransmission defects in the *dfmr1*-null condition include elevated

spontaneous vesicle fusion and increased vesicle exocytosis mediating glutamate release [36, 83, 88]. Targeted presynaptic reintroduction of dFMRP in *dfmr1*-null animals did not rescue these functional defects [88], indicating that the requirement for dFMRP is either postsynaptic or transsynaptic with respect to functional synaptic properties. Recent studies of the olfactory learning and memory circuit reinforce these findings. Transgenic RNAi against *dfmr1* specifically expressed postsynaptically in the MB impairs LTM after massed training but not when expressed in the presynaptic projection neurons [32]. Thus, presynaptic dFMRP is sufficient to promote proper synapse formation and architectural sculpting, whereas postsynaptic dFMRP is necessary to enable the concomitant modifications driving normal functionality. A precedent for this type of spatially separable functionality can be found in studies of the FMRP target PP2A, which in *Drosophila* mediates normal synaptic growth by working in the presynaptic compartment and normal levels of evoked transmitter release by working in the postsynaptic compartment [98].

## Conclusions

FMRP functions as a protein synthesis switch that modulates neuronal architecture and connectivity with considerable temporal and spatial specificity, thus facilitating differential behavioral outputs. FMRP regulates neural circuit development during critical windows of synaptogenesis and refinement and thus shapes mature neural circuit connectivity as well as functionality. FMRP function is regulated by activity, including sensory input and local synaptic transmission, and acts downstream of that activity to regulate appropriate connectivity. In a feedback loop, FMRP also coordinately regulates neuronal activity to modulate both membrane excitability characteristics and synaptic transmission properties. These FMRP requirements are clearly critical for enabling cognitive abilities, including the ability to learn and remember. However, FraX is a broad-spectrum disease with many abnormalities. A new insight is dysfunction in the regulation of daily biological rhythms. Defects in the circadian cycle are associated with sleep disruption and inappropriate periods of activity, which may be exacerbating or potentially causative, with respect to FraX cognitive defects.

What remains to be determined is how these requirements intersect. First, it will be critical to define the period (s) of FMRP requirement in establishing circuit architecture and connectivity in both circadian and learning/memory circuits. This understanding will allow molecular examinations during the most relevant developmental windows. Second, identification of circuit-specific alterations in FMRP targets, and particularly their activity-dependent regulation, should provide key insights into alternative modulatory approaches to correct these defects. Finally, elucidating the potentially shared signaling pathways regulating FMRP function should prove beneficial in therapeutic design to coordinately target related deficits. With regulation of circadian rhythmicity and learning/memory likely to be reciprocal and thus mutually reinforcing, FMRP may well provide a pivotal link in their interdependence.

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