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From FMRP Function to Potential Therapies for Fragile X Syndrome

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Abstract

Fragile X syndrome (FXS) is caused by mutations in the *fragile X mental retardation 1 (FMR1)* gene. Most FXS cases occur due to the expansion of the CGG trinucleotide repeats in the 5′ untranslated region (UTR) of *FMR1*, which leads to hypermethylation and in turn silences the expression of FMRP (fragile X mental retardation protein). Numerous studies have demonstrated that FMRP interacts with both coding and non-coding RNAs and represses protein synthesis at dendritic and synaptic locations. In the absence of FMRP, the basal protein translation is enhanced and not responsive to neuronal stimulation. The altered protein translation may contribute to functional abnormalities in certain aspects of synaptic plasticity and intracellular signaling triggered by Gq-coupled receptors. This review focuses on the current understanding of FMRP function and potential therapeutic strategies that are mainly based on the manipulation of FMRP targets and knowledge gained from FXS pathophysiology.

Keywords

Fragile X syndrome; *FMR1*; FMRP; Gq-coupled receptors; LTD; LTP; mouse model; RNA-binding protein; translation; therapeutic development

Introduction

FXS is Caused by Mutations in *FMR1*

Fragile X syndrome (FXS), also referred to as Martin-Bell syndrome or Escalante's syndrome, is the most common form of inherited intellectual disability (or mental retardation) and autism [1,2]. The incidence of FXS in males is approximately 1 in 2500 to 5000 and in females is 1 in 4000 to 6000 [2]. The cytogenetic discovery of the "fragile site" on the X chromosome in patients [3] and the higher incidence in males strongly suggest FXS as a genetic disease. Positional cloning definitively demonstrates the link between mutations

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in the *fragile X mental retardation 1 (FMR1)* gene, whose chromosome locus is at the Xq27.3 fragile site, and FXS [4]. Most FXS patients have a significant expansion of the CGG trinucleotide repeats in the 5′ untranslated region (UTR) of the *FMR1* gene. While healthy individuals have 5 to 45 CGG repeats (commonly 29-30 repeats), the affected individuals with full mutation normally have more than 200 repeats [5,6]. A few cases with missense mutations and deletions in the *FMR1* gene have also been reported [7-9].

Expansion of the CGG repeats correlates with the significant reduction or lack of expression of the *FMR1* gene product FMRP (fragile X mental retardation protein). It has been demonstrated that the altered FMRP expression in FXS patients with >200 CGG repeats may be mediated by different mechanisms. Some studies show that a high number of the CGG repeats may facilitate hypermethylation on the cytosine residues in the proximal regions of *FMR1*, including the promoter (i.e., about 250 bp to 1 kb upstream of the CGG repeats), leading to transcriptional silencing and consequently lack of protein translation [10,11]. Interestingly, in some alleles with >200 CGG repeats, there is only partial or no increase in methylation [12]. Cells from FXS patients that lack DNA methylation in the *FMR1* promoter exhibit normal or even higher levels of *FMR1* transcript [13,12]. Nevertheless, the level of FMRP is significantly reduced in FXS samples as compared to the samples from unaffected individuals [12,14], indicating that the expanded CGG repeats in the 5′ UTR may also affect translation efficiency [15]. Other mechanisms posit that full mutations in CGG repeats affect histone modification (including acetylation and methylation) [16,17] and may in turn suppress the activity of the *FMR1* promoter.

Animal Models of FXS

The development of valid animal models has been crucial for understanding FXS etiology, the function of FMRP, and has been invaluable in developing potential therapeutics for FXS. The main animal models of FXS have been generated with mouse [18], fruit fly [19,20], and zebrafish [21], in which the genetic ortholog of human *FMR1* is deleted. In another mouse model, the wild type *Fmr1* allele was mutated to harbor an isoleucine to asparagine mutation (I304N, corresponding to the I367N mutation in a rare FXS patient) [22,7]. It is important to note that the mouse model with an engineered expansion in CGG repeats does not show hypermethylation and lack of FMRP expression [23]. Thus, animal models with perfect construct validity are not available. Stem cells from FXS patients show *FMR1* silencing due to DNA hypermethylation upon differentiation [17], and can be used for drug screening and preliminary examination of the gene reactivation therapies [24,25].

Behavioral and physiological examinations have demonstrated that the current animal models show robust if not complete face validity of FXS. Some of the therapeutic strategies, which attenuate certain FXS-related symptoms in the animal models, have now been extended to human clinical trials, indicating reasonable predictive validity. FXS is characterized by mild to severe intellectual disability, susceptibility to seizures, hyperactivity, hypersensitivity to sensory stimuli, and autistic traits such as social anxiety, attention deficit, hand biting or flapping (repetitive behavior), and poor eye contact. Physical manifestations include long facial features with protruding ears, soft skin, connective tissue problems, and large testicles (macroorchidism). Many of these symptoms are recapitulated

in the *Fmr1* knockout (KO) mouse (Table 1). *Fmr1* KO mice show cognitive deficits when examined by Morris water maze ([26,27] but also see [28]), passive avoidance [29-31], contextual fear conditioning ([28] but also see [32]), and object recognition [33,34]. Susceptibility to seizures in *Fmr1* KO mice is implicated by wild-running and onset of seizure after receiving a high intensity siren (e.g. 125 dB at 1800-6300 Hz) [35,36]. In addition to audiogenic seizures (AGS), *Fmr1* KO mice also show enhanced limbic epileptogenesis and mossy fiber sprouting following a kindling paradigm [37]. Furthermore, electrophysiological studies have identified prolonged epileptiform discharges in the *Fmr1* KO hippocampus [38]. *Fmr1* KO mice are hyperactive and have more locomotor movement in the open field test [30]. They also show more entries to and spend more time in the center area of the open filed arena [30,39], indicating less anxiety (in contrast to the human FXS phenotype). However, in a modified open field chamber surrounded with mirrored walls, *Fmr1* KO mice avoid the center area [40]. Interestingly, independent groups have found that *Fmr1* KO mice show more [41], normal [42], or less anxiety [43] in the elevated plus maze test. Hyperarousal and sensorimotor gating phenotypes have been examined by acoustic startle responses and prepulse inhibition (PPI), respectively. While some studies show that low intensity white noise (at 80 dB) elicits higher startle responses but high intensity stimuli (at 120 dB) cause less startle in *Fmr1* KO mice [42,44], other studies demonstrate that deletion of *Fmr1* gene in mouse causes no change or lower startle in response to different levels of auditory stimuli [45,46]. Reduced PPI (a symptom observed in human FXS patients) [47] is seen in some investigations using *Fmr1* KO mice [48,49], while other reports have described increased PPI [35,42,45,47,46]. Autism-related symptoms are also detected in *Fmr1* mutant mice [46]. *Fmr1* KO mice show less social dominance than wild type animals in the social dominance tube test [40,50]. *Fmr1* mutants are less interested in social novelty and social interaction [46,43,51,33]. Defective communication (tested by ultrasonic vocalization)[52] and repetitive behavior (tested by marble burying) [53] are also detected in *Fmr1* KO mice [54-56]. FXS model mice harboring the I304N mutation exhibit hyperactivity, decreased acoustic startle response, repetitive behavior, and audiogenic seizures [22]. In addition to the neurological disorders, both *Fmr1* KO and I304N mutant mice show enlarged testes (i.e., macroorchidism) [18,22]. Furthermore, increased spine density and immature spines are observed from postmortem FXS samples [57,58], and such cellular abnormalities are detected in different brain regions of *Fmr1* KO mice [31,33,59,58] as well as in cultured mutant hippocampal neurons [49].

In addition to vertebrate models, *Drosophila* (fruit fly) has been successfully used to study FXS. Flies with mutations in *dfmr1*, whose gene product shows similar function to that of human FMRP [60], show altered synaptic structure [20], altered social interaction [61], impaired circadian rhythms [61], and defective cognitive function [62]. Additionally, *dfmr1* mutants have been used to validate therapeutic efficacy [62], understand signaling dysregulation in FXS, and screen potential pharmacological compounds for FXS therapy [63].

Although these animal models do not recapitulate all FXS symptoms and inconsistent phenotypes have been reported, it is evident that the current animal models do show reasonable face validity. The inconsistent behavioral phenotypes observed with *Fmr1* KO

mice may be due to differences in the experimental protocol, age [64], animal handling (or environmental factors such as different housing facilities and stress [65]), and genetic background [26,46,44,28]. Here, we would like to suggest that these inconsistent observations with animal models might reasonably reflect the fact that human FXS patients do not necessarily display a full spectrum of the symptoms. Importantly, differences in genetic background and environmental factors possibly contribute to the fact that FXS patients do not respond equally to behavioral and medical treatment [66,67].

Role of FMRP in mRNA metabolism

RNA Binding Activity of FMRP

FMRP is expressed in many tissues, but is most abundant in the brain and in the testis. In addition to its expression in the neuronal cell body, FMRP is also detected at dendrites and synapses [68]. Sequence analysis of FMRP reveals several RNA binding domains, which mediate FMRP-RNA interaction [69,70], implicating its function in regulating RNA metabolism. Cellular fractionation experiments demonstrate that FMRP co-sediments with actively translating polyribosomes [71-73], further suggesting its role in regulating mRNA translation.

Among the three canonical RNA binding domains, the two centrally localized hnRNP K-homology KH domains bind to the “kissing complex” tertiary motifs in RNA. The RGG (arginine-glycine-glycine) box is located close to the C terminus and binds to the G-quartet structures in RNA. The I367N missense mutation discovered in a human patient with severe FXS symptoms maps to the RNA binding pocket of the KH2 domain [69]. FMRP with the I367N mutation fails to bind to RNA [69] and polyribosomes [72]. The endogenous I304N-FMRP in mutant mice also does not show robust association with polyribosomes [22]. This suggests that the loss of RNA-binding/translation-regulating function of FMRP may be causal for the phenotypes in FXS.

RNA selection experiments *in vitro* have revealed that the KH2 domain of FMRP binds to an RNA complex called loop-loop pseudoknot or “kissing complex”. This binding activity is abolished in I304N-FMRP [74]. Further, RNA containing the “kissing complex” but not G-quartet decreases the association of FMRP with polyribosomes [74], suggesting that the “kissing complex” mimics the site that FMRP uses to regulate translation of its target mRNA. However, it is important to note that the kissing complex structure has not been yet convincingly identified in endogenous mRNAs.

The C- terminal RGG box has been found to bind to G-quadruplex RNA secondary structures *in vitro* [75]. Several FMRP target mRNAs (such as *Fmr1*, *Map1b*, and *Sema3f*) possess the predicted G-quadruplex structures, and *in vitro* biochemical examinations have confirmed their binding to FMRP [1]. A new structure SoSLIP (Sod1 stem loops) in *Sod1* (superoxide dismutase 1) mRNA may also interact with FMRP via the RGG domain [76].

In addition to the RNA binding domains, FMRP possesses two other regions (i.e., nuclear localization signal and nuclear export signal) that enable it to shuttle between the cytoplasm and the nucleus [77]. It is postulated that FMRP may pick up its mRNA targets in the

nucleus and transport them to the dendrites, where the local protein synthesis is regulated in an activity-dependent manner. It is shown that some protein-protein interaction domains may also exist to mediate FMRP association with proteins involved in translational regulation [78] and the RNA-induced silencing complex (RISC) [79,80].

RNA Targets of FMRP

It is estimated that FMRP binds to roughly 4% of the mRNAs in the brain [1]. An earlier genome-wide microarray study identified 432 FMRP-interacting mRNAs [81]. A recent study using UV cross-linking to covalently link FMRP to mRNA followed by stringent coimmunoprecipitation and high throughput sequencing identified 842 mRNA targets of FMRP in postnatal 11-25 day mouse brain. Many of the targets are involved in synaptic function, cell signaling, neural development, and autism. Most of the FMRP binding sites are in the coding region but not in the 5′ or 3′ UTR of the mRNA targets and no specific sequence or structural feature has been identified as preferred FMRP-binding motifs [82].

Another recent study used 4-thiouridine (4SU) photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) in HEK293 cells expressing HA-tagged FMRP. Complementary DNA libraries were generated and sequenced. By analyzing the resulting reads, the study identified around 6000 mRNAs that are bound to FMRP. More than 95% of the binding sites were either in the coding region or the 3′ UTR. Unlike the previous studies, this study identified only slightly more binding sites in the coding region than in the 3′ UTR. Further, two RNA-recognition elements (RRE) were identified as ACUK and WGGA (where K is G or U and W is A or U), which were found to occur in more than 50% of the binding sites [83]. Consistent with the study by Darnell et al. [82], many of the identified FMRP targets are also involved in autism spectrum disorder and synaptic signaling. It remains unclear whether these FMRP targets are functionally regulated by FMRP and contribute to cellular and behavioral abnormalities in FXS. Among all the identified FMRP targets, only a handful of them are verified by independent methods and functional studies (see Table 2).

Interestingly, FMRP also interacts with non-coding microRNAs (miRNAs) [84,79]. Among them, miR-125a and 125b can cooperate with FMRP to regulate the translation of validated FMRP targets PSD-95 and NR2A, respectively [84,85]. Further, overexpression of miR-125b results in longer and thinner spine [84], which is a cellular phenotype of FXS [58]. One functional significance of FMRP-miRNA interaction is that FMRP may regulate translation through coordination with miRNAs that binds to the 3′UTR of FMRP target mRNAs.

Function of FMRP in Regulating mRNA Metabolism

The existence of RNA binding domains in FMRP suggests its function in regulating RNA metabolism. Although some studies have demonstrated that FMRP regulates mRNA transport [86] and stability [87], it is well accepted that FMRP mainly suppresses the translation of its target mRNAs. First, in addition to mRNAs, FMRP also interacts with proteins such as CYFIP1 that regulate translation [78]. Second, FMRP has been found to co-sediment with actively translating polyribosomes particularly in synaptic preparations

[71,72]. Many FMRP target mRNAs such as Arc/Arg3.1, CaMKIIα, and PSD-95 localize in dendrites and in dendritic spines. In *Fmr1* KO neurons, MAP1B [88] and PSD-95 mRNAs [85] are more enriched in the actively translating polyribosomes rather than in the translationally quiescent messenger ribonucleoprotein (mRNP) complexes. Consequently, the expression of many FMRP targets is up-regulated in the absence of FMRP (see Table 2). It is thus believed that FMRP controls local protein synthesis at synapses by acting as a translational repressor and the loss of such translation control leads to many of the deficits seen in FXS. In a rare case, FMRP binding to *Sod1* mRNA positively regulates its translation [76]. It is important to point out that the protein levels of many FMRP targets remain unchanged or even reduced in *Fmr1* KO mice (see Table 2). To fully understand how the expression level of FMRP targets is controlled, investigations on compensatory mechanisms and secondary effects are needed.

Considering that FMRP interacts with the translation machinery and some of the FMRP targets (such as S6K1, PI3K, PIKE, and ERK1/2) can indirectly stimulate translation, it is understandable that the basal level of global protein synthesis is increased in *Fmr1* KO brain and in cell cultures derived from FXS patients [31,89,90]. In addition to its possible involvement in cap-dependent initiation and elongation steps [82], FMRP may also impede translation through coordination with miRNAs that bind to both FMRP and the 3′UTR of FMRP targets. The interaction between FMRP and components of the RISC (such as AGO1/2 and Dicer) may provide another layer of control on RNA metabolism. For example, the expression of FMRP targets NR2A is regulated through the coordination of FMRP and miR-125, and knockdown of AGO1 increases NR2A expression [84].

As the basal translation is elevated in FXS, activity-dependent up-regulation of translation is dampened in *Fmr1* KO neurons. As opposed to wild type neurons, *Fmr1* KO neurons do not show increased translation following the activation of mGluR1/5 [91,85,92], NMDAR [93], and upon membrane depolarization [94]. Consequently, certain aspects of mGluR1/5- and NMDAR-dependent synaptic plasticity (such as long-term depression and long-term potentiation) are altered in *Fmr1* KO mice [95,96].

Role of FMRP in Regulating Synaptic Function: the mGluR theory and beyond

Exaggerated mGluR1/5-LTD in *Fmr1* KO Mice

Synaptic protein synthesis and spine development are altered in FXS, thus it is hypothesized that FMRP regulates synaptic function and plasticity. Huber et al. showed that synaptic long-term depression (LTD) triggered by mGluR1/5 agonist DHPG is enhanced at the CA1 synapses in *Fmr1* KO mice [97]. This seminal study builds the foundation of the “mGluR theory”, which highlights that mGluR1/5 signaling is exaggerated in FXS, and explains how FMRP function is connected to mGluR1/5-mediated synaptic responses [95]. Multiple lines of evidence support the functional link between FMRP and mGluR1/5-LTD. First, mGluR1/5-LTD depends on new protein synthesis. The up-regulation of protein translation following mGluR1/5 activation may be related to the dynamic changes in FMRP. The activation of mGluR1/5 triggers rapid increase in FMRP translation [68] but it is followed

by FMRP degradation and de-phosphorylation, which may cause un-repression on the translation of certain synaptic molecules [92,98,99]. Indeed, the translation of several FMRP targets is up-regulated following DHPG stimulation (see Table 2). Second, the expression of mGluR1/5-LTD requires AMPA receptor internalization [100]. DHPG-stimulated receptor internalization depends on the translation of certain "LTD" proteins such as Arc and STEP. In the absence of FMRP, the translation of these "LTD" proteins would be less suppressed. The elevated expression of such "LTD" proteins in *Fmr1* KO neurons consequently facilitates AMPAR internalization [101] leading to enhanced synaptic depression. It is evident that other mGluR1/5-mediated synaptic functions are also regulated by FMRP. The activation of mGluR1/5 stimulates spine growth [102] and there are more immature spines in FXS neurons [57]. Collectively, mGluR1/5-mediated synaptic function and cellular changes are regulated by FMRP and exaggerated in FXS.

Effects of mGluR inhibition on FXS

Based on the mGluR theory, it is postulated that dampening mGluR1/5 activity may be therapeutic for FXS. Indeed, administration of mGluR5 antagonists to animal models of FXS has shown promising therapeutic effects. Specifically, administration of mGluR5 antagonist MPEP attenuates the elevated protein translation [89,90], the enhanced AMPAR internalization [101], the abnormal spine morphology and PPI ([49] but also see [54]), AGS, hyperactivity [39], and repetitive behavior [54] in *Fmr1* KO mice. The use of fenobam, a potent negative allosteric modulator of mGluR5, promisingly attenuates spine abnormality [49] and impairments in procedure memory and avoidance discrimination [103]. Genetic approaches have further demonstrated the therapeutic role of mGluR1/5 in FXS. Double mutant mice (heterozygous for *mGluR5* and hemizygous for *Fmr1*) show normal basal translation, spine density, no significant AGS, and normal fear memory extinction. However, macroorchidism is not rescued [31]. Intriguingly, a more recent study found that the *mGluR5/Fmr1* double mutants still show AGS, repetitive behavior, and abnormalities in anxiety and memory [45]. Genetic reduction of mGluR1 also fails to correct the major symptoms of FXS [45].

Encouraged by the effects of mGluR5 inhibition, an open label single dose fenobam trial has been performed with 12 adult patients. While there is mild improvement in PPI, no significant effect is observed for CPT (continuous performance test) [104]. Because no adverse reaction was identified, this study warrants further investigation on the therapeutic effect of fenobam. In addition to the available mGluR5 antagonists, new compounds are being developed and tested in clinical trials. These include AFQ056 and CTEP that rescue the enhanced mGluR1/5-LTD, the enhanced protein synthesis, spine morphology, cognitive deficits, hypersensitivity, PPI, and social interaction phenotypes in *Fmr1* KO mice [48,105,106]. In a phase II double blind placebo-controlled crossover trial with AFQ056 on 30 adult males for 28 days, administration of AFQ056 resulted in improvement of maladaptive behaviors only in a selective subpopulation of FXS patients with full promoter methylation [67]. Better understanding on the therapeutic value of mGluR5 antagonists may need larger scale trials. Furthermore, the development and clinical trials of other mGluR5 negative allosteric modulators such as STX107 (Seaside therapeutics) and Ro4917523

(Hoffman La-Roche) should aid in understanding the efficacy of mGluR antagonists for the treatment of FXS.

mGluR-Independent Mechanisms

Inhibition of mGluR1/5 rescues some but not all FXS-related symptoms [45] and shows therapeutic effects on a sub-population of FXS patients [67]. This suggests the existence of mGluR-independent mechanisms. Similar to mGluR1/5, activation of another group of Gq-coupled receptors, such as Gq-coupled muscarinic acetylcholine receptors (Gq-mAchR), also triggers AMPAR internalization and translation-dependent LTD. Gq-mAchR-LTD is significantly exaggerated in *Fmr1* KO mice [107]. Treating *Fmr1* KO mice with an inhibitor of M1 (one subtype of Gq-mAchR) dampens AGS [108]. It remains to be determined whether simultaneous blocking mGluR1/5 and Gq-mAchR offers more robust correction of FXS traits. Moreover, several lines of evidence have demonstrated that alteration of other G-protein coupled receptors is connected to FXS. For example, genetic or pharmacological inhibition of the Gi-coupled muscarinic M4 receptor rescues limited abnormal behaviors in *Fmr1* KO mice [109,110]. It was also shown that dopaminergic D1 receptor-mediated AMPAR surface expression and signaling in prefrontal cortex require FMRP, and the D1 receptor agonist SKF81297 attenuates hyperactivity in *Fmr1* KO mice [111]. More recently, Costa et al. found a functional cross-talk between 5-HT7 serotonin receptors and mGluR1/5 in *Fmr1* KO neurons. Pharmacological activation of 5-HT7 receptor suppresses enhanced mGluR1/5-LTD and AMPAR internalization [112].

In addition to the altered Gq-LTD, abnormal synaptic long-term potentiation (LTP) is also observed in *Fmr1* mutants. Studies have shown that FMRP may regulate both the mGluR1/5 and the NMDAR components of LTP. In the wild type visual neocortex, LTP is slightly attenuated by the NMDAR antagonist CPP and severely suppressed by the mGluR1/5 antagonists MPEP and MCPG [113]. The degree of LTP in MPEP-treated slices from wild type mice is similar to that from *Fmr1* KO mice without MPEP treatment. These surprising results indicate that the mGluR1/5 function is dampened rather than enhanced in the neocortex of *Fmr1* mutants. Further, treating slices from *Fmr1* KO mice with MPEP does not correct the LTP deficits. Additionally, Suvrathan et al. found that certain aspects of mGluR1/5-dependent LTP are impaired in the amygdala of *Fmr1* KO mice, and application of MPEP fails to correct the impairment [114].

In the hippocampus, LTP induced by the application of glycine depends on both NMDAR and mGluR1/5, and is also impaired in *Fmr1* mutants. Interestingly, lower LTP in *Fmr1* mutants is slightly dampened by the mGluR1/5 antagonist DL-AP-3 but not by the NMDAR antagonist APV [115]. The involvement of FMRP in regulating NMDAR function has also been suggested by several other studies. Lauterborn et al. reported that LTP induced by weak theta burst stimulation (TBS) at the threshold value (5 burst) is dramatically defective at the CA1 synapses in *Fmr1* KO mice [96]. This complements an earlier study documenting that LTP induced by a stronger TBS (10 burst) is normal in the *Fmr1* mutant hippocampus [28]. The intensity-dependent LTP phenotypes suggest that the learning and memory deficits in *Fmr1* mutants may also depend on the intensity of the behavioral training protocols, and may help explain some inconsistent behavioral results from different labs. Moreover,

NMDAR hypofunctions such as lower NMDAR-mediated synaptic transmission and reduced NMDA/AMPA ratio are observed in the dentate gyrus (DG) of *Fmr1* mutants [116,117]. Both NMDAR-mediated LTP and LTD at the DG synapses are also impaired in the absence of FMRP [116].

In addition to postsynaptic function, several lines of evidence suggest that FMRP may also regulate presynaptic events. First, FMRP immunoactivity is detected in axons and presynaptic terminals [118]. It is proposed that FMRP may regulate the localization and translation of mRNAs encoding presynaptic proteins [119]. Indeed, proteomic studies have found increases in presynaptic proteins (such as Rab-3A, synapsin-1, and synaptophysin) in *Fmr1* KO neurons. Ultrastructural and physiological examinations reveal immature presynaptic terminals and impaired paired pulse facilitation (PPF) at the Schaffer collateral-CA1 synapses of *Fmr1* mutants [120]. An independent study failed to observe the PPF deficits, but found that Ca^{2+} influx in presynaptic neurons and synaptic vesicle recycling are enhanced in *Fmr1* KO mutants [121]. Furthermore, presynaptic FMRP may also regulate presynaptic cytoskeleton and the motility of axon growth cone [122]. Interestingly, these altered presynaptic functions in FXS may be mediated through mechanisms independent of translation regulation. A recent study by Deng et al. demonstrates that protein-protein interaction between FMRP and presynaptic BK (the large conductance Ca^{2+}-activated potassium) channels modulates action potential and neurotransmitter release [123]. FMRP also regulates the gating of the Na^{+}-activated potassium channel Slack through direct protein-protein interaction [124].

Effects of Manipulating FMRP Targets on FXS

As the basal level of translation is elevated in *Fmr1* KO neurons, it is generally accepted that FMRP suppresses the translation of its target mRNAs. Thus, it is postulated that the elevated expression of certain “key” FMRP targets may be causal for FXS, and dampening such “key” targets may be therapeutic. However, this therapeutic approach is still in its infancy. Only a handful of studies show that suppressing FMRP targets attenuates cellular abnormalities [125,93] and certain (but not all) behavioral phenotypes of FXS [50,41,126]. Most of the verified FMRP targets are involved in synaptic plasticity, neurotransmission, and neuronal signaling. Some targets have a connection to neurological and psychiatric disorders. The therapeutic value of FMRP targets is investigated using a combination of genetic and pharmacological approaches.

Arc (activity-regulated cytoskeleton-associated protein), an FMRP target, is associated with the synaptic cytoskeleton network and regulates AMPA receptor (e.g. GluR1) trafficking [100,125]. Its involvement in FXS is implicated by the observation that the basal level of Arc expression is elevated in *Fmr1* KO neurons [98]. Further, Arc translation is rapidly stimulated by the activation of mGluR1/5 and is required for mGluR1/5-LTD [100,125]. Genetic deletion of Arc in wild type and *Fmr1* KO mice results in no significant mGluR1/5-LTD [125]. Another FMRP target, APP (amyloid beta precursor protein) [127] is also a structural protein that regulates synaptic function [128] as well as neurodegeneration. While overexpression of APP in *Fmr1* KO mice increases seizure susceptibility [129], reduction of APP in *Fmr1* KO mice rescues multiple FXS symptoms including AGS, higher density of

immature spines, and the enhanced mGluR1/5-LTD [126]. These studies identify a link between FXS and Alzheimer's disease (AD) [130], and suggest that therapies developed for AD to reduce APP level may be used to treat FXS. Interestingly, Arc expression level is also significantly higher in AD patients, and genetic deletion of Arc reduces amyloid-beta level in a mouse model of AD [131]. The expression level of another FMRP target, STEP (striatal-enriched tyrosine phosphatase), is elevated in *Fmr1* KO neurons as well as in AD patients. Strikingly, genetic reduction of STEP rescues certain FXS phenotypes [50] and also reverses cognitive impairment in AD mice [132].

A number of FMRP targets are functionally involved in the PI3K (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase) pathways (see Table 2), which positively regulate ribosomal function and translation in an activity-dependent manner. It is also important to note that the activity of both PI3K and MAPK is required for LTP and LTD. In *Fmr1* KO neurons, PI3K activity and the expression of its catalytic subunit p110 are elevated [90,133]. Inhibition of PI3K by LY294002 suppresses the cellular phenotypes of FXS including exaggerated basal translation, GluR1 internalization, and spine density [90]. Increased expression of an up-stream activator of PI3K (i.e. PIKE or PI3K enhancer) and increased activity of a down-stream effector of PI3K (i.e. mTOR or mammalian target of rapamycin) are observed in the hippocampus of *Fmr1* KO mice. Furthermore, activity of an up-stream suppressor of PI3K (i.e. PTEN or phosphatase and tensin homolog) is decreased in the hippocampus of *Fmr1* KO mice [133]. However, inhibition of mTOR with rapamycin only suppresses mGluR1/5-LTD in wild type [134] but not *Fmr1* KO mice [133]. Treating *Fmr1* KO mice with rapamycin does not suppress the elevated basal translation, but does dampen AGS [89]. Homer1, another FMRP target, is an adaptor protein that connects mGluR1/5 and the PI3K signaling cascade [135]. Although Homer1 mRNA interacts with FMRP, its expression is unchanged in *Fmr1* KO neurons [136]. Intriguingly, mGluR5 associates less with the long Homer isoforms but more with the short Homer1a in *Fmr1* KO mice [136,137]. Genetic deletion of Homer1a in *Fmr1* KO mouse decreases AGS and center occupancy in the open field test without affecting the enhanced mGluR1/5-LTD and basal translation [137]. As mentioned earlier, some FMRP targets are related to autism. The mRNA of TSC2, which regulates mTOR activity, binds to FMRP. Genetic mutation of *Tsc2* is linked to TSC (tuberous sclerosis complex) disease that displays symptoms of autism and mental retardation. *Tsc2* heterozygous KO mice show reduced mGluR1/5-LTD and protein synthesis. Genetic reduction of *Tsc2* in *Fmr1* KO mice normalizes mGluR1/5-LTD and memory deficits to the wild type level [138].

ERK1/2 (extracellular signal-regulated kinase 1/2), a component of the MAPK pathway, is an FMRP target. The activity rather than the expression level of ERK1/2 is elevated in *Fmr1* KO mice. Pharmacological inhibition of ERK1/2 reduces AGS, elevated protein translation, and prolonged epileptiform discharges in *Fmr1* KO mice [38,89]. Osterweil et al. reported similar therapeutic effects in *Fmr1* KO mouse by using lovastatin, (a clinically approved cholesterol-lowering drug) to suppress ERK1/2 activity [139]. Several molecules up- and down-stream of ERK1/2 are also FMRP targets. PAK1 (p21-activated kinase 1) is a positive regulator of ERK1/2 and plays an important role in spine morphology. In addition to its mRNA, PAK1 protein also interacts with FMRP. Expression of dominant negative PAK1 in

Fmr1 KO mice rescues the higher spine density, cortical LTP deficits, hyperactivity, repetitive behavior, and impairment in trace fear conditioning [59]. Additionally, significant therapeutic effects can also be observed in *Fmr1* KO mice using a potent PAK inhibitor [140].

Although studies on the therapeutic function of ERK1/2 and PI3K have shown some degree of controversy [90,89], the two signaling pathways may converge and co-regulate the activity of some FMRP targets. S6K1 (ribosomal protein S6 kinase 1) is involved in ribosome biogenesis and regulates protein translation; its activity can be up-regulated by ERK1/2- and PI3K-mediated phosphorylation at different residues [141]. Although the expression level of S6K1 is normal in *Fmr1* KO neurons, phospho-S6K1 is elevated [133]. Genetic deletion of S6K1 in *Fmr1* KO mice rescues the enhanced mGluR1/5-LTD, abnormal spine morphology, and deficits in recognition memory and social interaction. However, some FXS-related phenotypes such as hyperactivity and repetitive behavior are not corrected [33]. GSK3 is another FMRP target whose activity can be down-regulated by PI3K- [142] and ERK1/2-mediated phosphorylation [143]. Although PI3K and ERK1/2 activity are elevated in *Fmr1* KO mice, the phosphorylation of both GSK3 and GSK3 is decreased in FXS [144]. Thus GSK3 activity is abnormally higher in *Fmr1* KO neurons. Treating *Fmr1* KO mice with GSK3 inhibitors lithium and SB-216763 attenuates AGS, hyperactivity, defective cognitive function (such as passive avoidance memory, contextual, and cued fear memory), deficits in social interaction (such as social preference and social anxiety), defective neurogenesis, and abnormal spine morphology in cortical and newborn hippocampal neurons [144-146,51,43]. An open label study with 15 FXS males treated with lithium showed that lithium improves social and maladaptive behavior as well as auditory memory. However, 7 individuals had side effects of polydipsia and polyuria [66].

Another group of FMRP targets includes voltage-gated potassium channels (Kv3.1 and Kv4.2) [75,147,148,93], ligand-gated channels (such as GABA-A, AMPA, and NMDA receptors) [82,84,149], and G protein-coupled GABA-B receptors [82]. Kv4.2 plays important roles in regulating excitability, seizures, and plasticity. Lee et al. found that the Kv4.2 mRNA is localized in the dendrites, and its 3′ UTR binds FMRP and is required for FMRP-dependent translation suppression [93]. Functionally, inhibiting Kv4.2 rescues the defective LTP in *Fmr1* KO mice [93]. Intriguingly, Gross et al. found that FMRP facilitates the translation of Kv4.2, whose expression is decreased in *Fmr1* KO mouse brain [148]. A couple of studies show evidence that different subunits of GABA-A receptors [150-152] are down-regulated in *Fmr1* KO mice. It is not surprising that GABA-A receptor agonists and positive allosteric modulators (such as diazepam and ganaxolone), which are clinically used as anticonvulsants, attenuate AGS [153]. Another GABA system modulator, gaboxadol, reduces hyperactivity and PPI without affecting acoustic startle and cued memory in *Fmr1* KO mice [154]. Although GABA-B receptor mRNA is identified as an FMRP target by high-throughput screening [82], its expression level in FXS is not known. However, its functional relevance is demonstrated by the fact that GABA-B agonists baclofen and arbaclofen (STX209) rescue AGS in *Fmr1* KO mice [155,156]. Further, arbaclofen treatment reduces the enhanced basal translation, AMPA receptor internalization, and spine density in *Fmr1* KO mice [156]. A Phase 2 randomized, double blind, and placebo

controlled crossover trial with STX209 in 55 males and 8 females showed that STX209 treatment improves social behavior in all patients [157]. It has been demonstrated that the expression level of NMDAR subunits (i.e. NR1 and NR2B) is increased in the neocortex and hippocampus of *Fmr1* KO mice ([158], but also see [136] and [159]). FMRP also suppresses the translation of NR2A [84]. Thus, there is possibly a combination of excessive NMDAR function and deficient GABA function (but also see [116]). Based on this hypothesis, acamprosate, which acts as an NMDAR antagonist and GABA-A agonist, was used in an open-label clinical study. Young FXS patients receiving acamprosate show significant improvement in social behavior and reduction in hyperactivity [160].

Minocycline, an FDA-approved broad-spectrum tetracycline antibiotic, shows some promising therapeutic effects in human FXS patients. In a pilot open label study on 50 FXS individuals, minocycline treatment improved cognition, language and behavior [161]. In another open label study, 20 FXS individuals treated with minocycline showed improvement in irritability and other global behavior tests [162]. A double blind, placebo-controlled, crossover trial with minocycline treatment in FXS children resulted in improvements in anxiety and mood-related behavior [163]. Treating *Fmr1* KO neurons and mice with minocycline corrects abnormal spine morphology, anxiety phenotype, defective ultrasonic vocalization, AGS, and hyperactivity [41,56,164]. Possible mechanisms include minocycline-mediated inhibition of ribosome function [165] or MMP9 (matrix metallopeptidase 9), which is an FMRP target whose expression level is elevated in FXS [166,41].

Connecting the mGluR Theory and FMRP Targets

The mGluR theory and FMRP-mediated translation have been fundamental to the development of therapeutic approaches for FXS. It is not clear how the dys-regulated translation of FMRP targets contributes to elevated mGluR1/5 signaling in FXS. A recent study reported that the expression of mGluR5 itself, whose mRNA is identified as an FMRP target through high through-put screening [82], is slightly but significantly elevated in the prefrontal cortex of FXS patients [167]. However, another study has shown that the expression level of mGluR5 is not changed in the forebrain and cerebellum of *Fmr1* KO mice [136]. Although, as a major downstream effector of mGluR1/5 signaling, ERK1/2 shows higher activity in *Fmr1* KO neurons, its total expression level is not changed [139]. Thus, the changes in ERK1/2 may represent an outcome of the elevated mGluR1/5 signaling rather than having a causal role on the enhanced mGluR5 function. Investigation on the molecules in the PI3K cascade has found that the expression levels of both PIKE and the p110 subunit of PI3K are elevated in the absence of FMRP [90,133]. Considering that PI3K activation can be triggered by mGluR1/5 stimulation, the enhanced expression of these FMRP-targets may contribute to the elevated basal mGluR1/5 activity in FXS.

Future Directions

Since the positional cloning of the *FMR1* gene, there have been tremendous advances in understanding the function of FMRP, which have led to rational designs of therapeutic approaches. While new agents are being examined in animal models and clinical trials,

successful repurposing the available drugs such as memantine, acamprosate, minocycline, fenobam, baclofen, lithium, and lovastatin could benefit FXS patients without involving the lengthy drug development process. Because FMRP regulates many aspects of neuronal function, simultaneous manipulation of multiple FMRP targets and/or signaling pathways should also deserve significant consideration.

Although the prevailing theory posits that FMRP suppresses translation and mGluR1/5 signaling, significant involvement of FMRP in up-regulating protein synthesis, mGluR1/5-independent synaptic function, and protein-protein interaction have also been identified. It remains to be determined how these new functions of FMRP are relevant to FXS etiology.

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Table 1

Human FXS traits that are recapitulated in *Fmr1* knockout mice.

FXS symptoms in human	FXS-related behavior in Fmr1 KO mice
Intellectual disability	Defective spatial learning and memory [26,27] Defective passive avoidance memory [30,29] Defective contextual memory [28] Defective recognition memory [33,34]
Susceptibility to seizures	Audiogenic seizure [35,36] Enhanced limbic epileptogenesis [37] Prolonged epileptiform discharges [38]
Hyperactivity	Increased locomotor movement In open field test [30,43]
Hyperarousal	Higher acoustic startle responses to low intensity stimuli [42,44]
Abnormal sensorimotor gating (reduced PPI)	Variable phenotypes in PPI [48,49,35,42,45,47,46]
Social anxiety Defective social interaction	Deficits in social dominance [40,50] Decreased interests in social novelty and social interaction [46,43,51,33] Defective communication (tested by ultrasonic vocalization) [55]
Perseveration/repetitive behavior	Increased marble burying [56,22]
Macroorchidism	Enlarged testes [18,22]
Higher density of immature spine	Higher density of immature spine [31,33,59,58,49]

Table 2

FMRP targets that may be functionally involved in FMRP-regulated translation and FXS-related phenotypes

Gene symbol	Protein name	Validation method	Changes in FXS	Response to mGluR1/5 stimulation	Therapeutic effects	Inhibitors
Agap2	PIKE	HT [82]	↑protein [133]			
App	APP	HT [82,83], CoIP [127]	↑protein [126]	↑translation [127]	AGS, hyperactivity, spines, mGluR-LTD [126]	
Arc	Arc	HT [82,83], CoIP [168]	↑protein [168] ↓ protein [159]	↑translation [125,100]	mGluR-LTD [125]	
Camk2a	CaMKIIα	HT [82], CoIP [168,149]	↑protein [168,92]	↑translation [89,169,92]		KN62, KN93
Cyfip2	CYFIP2	HT [82,83]	↑protein [170]			
Dlg4	PSD-95	HT [82], miR-125a [85], G-quadruplex [91]	↑protein [91] ↓protein degradation [171] ↓ protein [159]	↑translation [85,91]		
EF1a	EF1α	CoIP [172]	↑protein [172]	↑translation [173]		
Fmr1	FMRP	In vitro [174]	↓ or lack of expression	↑translation followed by degradation [92,98,99,68]		
Gabbr1	GABA-B1	HT [82]			Protein synthesis, AMPAR internalization, abnormal spines, AGS [155,156]	STX209, baclofen
Gabrd	GABA-Aδ	In vitro [175]	↓protein [150]		AGS [153], hyperactivity and PPI [154]	Acamprosate, alphaxalone, gaboxadol, diazepam
Gria1	AMPA-1	HT, Co-IP [149]	↑protein [149,158] no change [136]	↑translation [149]		
Grin1	NR1	HT [82], CoIP [158]	↑protein [158] no change [136] ↓ protein [159]		Some clinical efficacy in human patients [160,176]	Memantine, Acamprosate
Grin2a	NR2A	miR-125b [84]	no change [136] ↓ protein [159]		Some clinical efficacy in human patients [160,176]	Memantine, Acamprosate
Grin2b	NR2B	HT [82], CoIP [158]	↑protein [158] no change [136] ↓ protein [159]		Some clinical efficacy in human patients [160,176]	Memantine, Acamprosate
Grm5	mGluR5	HT [82]	↑protein [167] no change [136]		mGluR-LTD [106,31], AMPA receptor internalization [101], protein synthesis [106,31,89,90], dendritic spines [31,49,106], visual cortical plasticity [31], AGS [106,31,39], hyperactivity [39], PPI [49,48], social interaction [105], repetitive behavior [54], body weight [31], macroorchidism [106], hypersensitivity [106].	MPEP, Fenobam, AFQ056, CTEP
Gsk3b	GSK3β	HT [82,83]	↑activity [144]		AGS, hyperactivity, passive avoidance memory, contextual, and cued fear memory, social	Lithium, SB216763

Gene symbol	Protein name	Validation method	Changes in FXS	Response to mGluR1/5 stimulation	Therapeutic effects	Inhibitors
					preference and social anxiety, defective neurogenesis, spine abnormality [144-146,51,43]	
Hcn1	HCN1		↑protein [177]			
Homer 1	Homer1	HT [83]		Unchanged [137]	Restored mGluR5 signaling, AGS, anxiety phenotype in open field [137]	
Kcnc1	Kv3.1; Kv3.2	In vitro [75]	↑protein [147]			
Kcnd2	Kv4.2	HT [82], in vitro [93]	↑protein [93], ↓ protein [148]		LTP [93]	Heteropodatoxin HpTx2
Map1b	MAP1B	HT [82,83] In vitro [88,178]	↑protein [168,88,92]	↑translation [179,92]		
Mapk1	MAPK1 or ERK2	HT [82,83]	↑phosphorylation [92,139]	↑activation [92,89]	Protein synthesis [89,139], AGS [89,139]	U0126, SL 327, lovastatin
Mmp9	MMP9		↑protein [166,41]		Spine abnormality, anxiety phenotype in elevate plus maze, AGS, hyperactivity, communication [41,56,164]	Minocycline
Mtor	mTOR	HT [82,83]	↑phosphorylation ↑activity [133]	↑activation [133]	AGS [89]	Rapamycin
Pak1	PAK1	HT [82,83]			Spine abnormality, LTP, hyperactivity, repetitive behavior, anxiety, trace fear conditioning, AGS [59,140]	FRAX486
Pik3cb	PI3K P110β catalytic subunit	HT [83],	↑protein ↑activity [133,90]	↑translation ↑activation [90]	Protein synthesis, spine abnormality, AMPA receptor internalization [90]	LY294002 Wortmannin
Pten	PTEN	HT [82,83]	↓phosphorylation [133]			
Ptpn5	STEP	HT [82]	↑protein [50]	↑translation [180]	AGS, social interaction, social anxiety [50]	
Rgs5	RGS5	In vitro [175]				
Rps6kb1	S6K1	HT [83]	↑phosphorylation [33]	↑activation [133]	Protein synthesis, mGluR-LTD, dendritic spines, novel object recognition, social interaction [33]	
Sapap3/4	SAPAP3/4		↑protein [158] ↓protein [159]			
Sema3f	SEMA3F	In vitro [181]				
Shank1	SHANK1	HT [82,83], 3'UTR [158]	↑protein [158]	↑translation [158]		
Shank3	SHANK3	HT [82]	↑protein [158]			
Sod1	SOD1	In vitro [76]	↓protein [76]			
Tsc2	TSC2	HT [82,83]			mGluR-LTD, memory deficit [138]	

HT: high through-put screening; CoIP: coimmunoprecipitation; AGS: audiogenic seizure; PPI: prepulse inhibition; ↑: increase; ↓: decrease. Therapeutic effects are observed in *Fmr1* KO mice when the corresponding FMRP target is genetically reduced or pharmacologically inhibited.