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REVIEW

Therapeutic Strategies in Fragile X Syndrome: From Bench to Bedside and Back

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Abstract Fragile X syndrome (FXS), an inherited intellectual disability often associated with autism, is caused by the loss of expression of the fragile X mental retardation protein. Tremendous progress in basic, preclinical, and translational clinical research has elucidated a variety of molecular-, cellular-, and system-level defects in FXS. This has led to the development of several promising therapeutic strategies, some of which have been tested in larger-scale controlled clinical trials. Here, we will summarize recent advances in understanding molecular functions of fragile X mental retardation protein beyond the well-known role as an mRNA-binding protein, and will describe current developments and emerging limitations in the use of the FXS mouse model as a preclinical tool to identify therapeutic targets. We will review the results of recent clinical trials conducted in FXS that were based on some of the preclinical findings, and discuss how the observed outcomes and obstacles will inform future therapy development in FXS and other autism spectrum disorders.

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Fragile X syndrome (FXS) is one of the first single gene disorders manifesting features of autism spectrum disorder (ASD) in which extensive study of the neurobiology and synaptic mechanisms of disease in cellular and animal models has been possible. The enormous progress in basic and preclinical and clinical translational work in FXS in the last several decades has allowed FXS to emerge as an important model to illustrate successes and hurdles in the development of future targeted treatments for autism and related developmental disorders. FXS is the most common known genetic cause of intellectual disability and ASD, with an estimated frequency of about 1 in 4000–5000 [1]. The disorder affects all ethnic groups worldwide.

Genetics and Phenotype of FXS

FXS is one of the fragile X-associated disorders (FXDs), all of which arise from a trinucleotide repeat (CGG) expansion mutation in the promoter region of *FMR1*. The CGG sequence is transcribed into the 5' untranslated region of *FMR1* mRNA and thus length of the repeat sequence does not affect the sequence of the protein product of *FMR1* [fragile X mental retardation protein (FMRP)] [2]. Small expansions in the gene (55–200 CGG repeats), termed the "premutation", occur in about 1 in 430–468 males and 1 in 151–209 females in the USA [3, 4], and is associated with risk for fragile X-associated tremor/ataxia syndrome and fragile X-associated primary ovarian insufficiency. Although the premutation is transcribed and translated to give FMRP, toxicity in fragile X-associated tremor/ataxia syndrome and fragile X-associated primary ovarian insufficiency is thought to occur through a mechanism related to elevated cellular *FMR1* mRNA levels and resultant CGG repeat-mediated RNA toxicity [2]. Large expansions in *FMR1* (>200 CGG repeats), termed the "full mutation," cause FXS, which results from methylation and transcriptional silencing of *FMR1* with consequent loss or significant reduction in expression of FMRP [2]. Expansion mutations in *FMR1* tend to increase in size as they are inherited through the generations, so fragile X-associated disorders affect families in multiple generations, with more individuals affected with FXS in later generations.

Males with FXS typically present with developmental delay, particularly language delay, and ultimately display intellectual disability that can range from mild to severe [5]. Hypotonia is often seen early in life and evolves into coordination and praxis problems in older children and adults. Physical features include macro-orchidism in most men, and variable presence of craniofacial characteristics, including prominent ears, macrocephaly, long face, prominent jaw and forehead, midfacial hypoplasia, and high arched palate. Loose connective tissue leads to hyperextensible joints, flat feet, and soft redundant skin on the palms [5]. Females with a full mutation are more variably and usually more mildly affected than males because of production of FMRP in cells that express the normal X chromosome with the nonmutated *FMR1* allele. The severity of cognitive impairment in females with a full mutation is inversely related to the activation ratio for the normal *FMR1* allele and levels of expressed FMRP [6]. Males with size (full and premutation) or methylation (partially unmethylated full mutation) mosaicism may be more mildly affected, with severity related to the percent of unmethylated *FMR1* alleles and FMRP levels.

A number of medical problems appear to be more prevalent in FXS than in the general population, based on prior small studies from individual or small groups of centers, and confirmed in a large dataset collected from a natural history study being conducted through the Fragile X Clinic and Research Consortium, a consortium of fragile X clinics in the USA [7]. These problems include seizures, strabismus, frequent otitis media, gastroesophageal reflux, and sleep apnea and other sleep disorders (Table 1).

Cognitive, Behavioral and Language Features of FXS

The majority of males with FXS will meet criteria for mild-to-severe intellectual disability [8]. The average IQ in men with FXS is 40–50, with a mental age of about of 5–6 years. Females with FXS are often less affected than males with about 25 % having cognitive impairment and others frequently being diagnosed with learning disabilities [9]. Average IQ in women is about 80, with a range from severe impairment to normal or even superior ability. There is a relatively consistent pattern of intellectual weaknesses (generally milder in severity in females) and strengths distinct to both males and females with FXS [10]. Relative weaknesses include visuospatial skills, working memory, processing of sequential information, and attention [10], while there are relative strengths in simultaneous processing and long-term memory.

Multiple studies have shown a decrease in full-scale IQ scores with age as children with FXS become older [11–13]. Standard scores on the Vineland Adaptive Behavior Scale for overall adaptive behavior as well as subdomains have also been shown to decline with age during childhood, in males more so than in females with FXS [14]. Decline in standard scores for intelligence and adaptive function is not the results of loss of skills or regression but rather failure to keep pace with the normal rate of intellectual development. Scores are felt to be fairly stable in adulthood.

The behavioral phenotype in males with FXS covers a wide spectrum, although there are certain behaviors that are very commonly seen. These behaviors fall into the broad categories of attention/hyperactivity, hyperarousal, anxiety, and aggression. As in other areas, females with FXS have the same spectrum of behavioral difficulties but with milder symptomology.

Attention disorders, including impulsivity, hyperactivity and inattention, are extremely common in FXS and result in a comorbid attention deficit hyperactivity disorder diagnosis in as many as 73 % of males and 30–63 % of females [10, 15–18]. Impulsivity in boys with FXS does not seem to improve with age, which means that this behavior has the

Table 1 Frequency (%) of medical problems in fragile X syndrome (FXS)

	FXS males (FXCRC)	FXS females (FXCRC)	FXS all (FXCRC)	FXS (past)	General population
Seizures	12	3	10	15	1
Frequent ear infections	55	46	53	56	14
Strabismus/lazy eye	18	13	16	30	3
Sleep problems	26	30	27	40	17
Sleep apnea	7	7	7	34	1
Gastroesophageal reflux	10	14	11	31	5

Data were adapted from Kidd et al. 2014 [7]. FXS past study data is taken from the largest study published before the Fragile X Clinic and Research Consortium (FXCRC) Database study potential to present long-term challenges to the individual and their caregivers [19].

Anxiety disorders have been reported in 86.2 % of males with FXS and 76.9 % of females with FXS [20]. Although individuals with FXS manifest high rates of generalized anxiety and of virtually all subtypes, the anxiety disorders most frequently found in the literature are specific phobias and social anxiety [10, 18, 20].

Hyperarousal, an over-reaction to sensory input, can be triggered in FXS by a wide range of situations, including noises, new environments, crowds, interpersonal distance, eye contact, and new people [21]. The effects of this hyperarousal are widespread, and include high levels of motor activity (e.g., running, jumping), stereotypic motor movements (e.g., hand-flapping), gaze aversion, and perseverative behaviors [21–23].

Aggression and self-injurious behavior are often linked to hyperarousal or anxiety. These problem behaviors are especially detrimental to family functioning as they can significantly impede an individual's ability to participate in daily activities. Self-injurious behavior typically appears fairly early in development; onset for most individuals (both male and female) occurs at 1–3 years of age [24]. Aggression typically occurs later, mostly during the pubescent and postpubescent period [25].

Language development is globally delayed for most individuals with FXS. It is not uncommon for individuals with FXS to remain prelinguistic communicators until much later in life than is seen in typical development [26]. However, the majority of males and females with FXS will obtain spoken language at some point, and they will continue to gain language skills throughout their lifespan, albeit at a slower pace than typically developing individuals [27, 28].

In general, individuals with FXS have stronger receptive than expressive language skills. While receptive language skills are usually below chronological age expectations, they are commensurate with nonverbal mental age [29], and receptive vocabulary, a major strength, can be well above mental age. However, individuals with comorbid FXS and autism have receptive language skills below their nonverbal mental age, and as autism severity increases so do the deficits in receptive vocabulary and grammar [30, 31]. Expressive language is delayed, with nonverbal mental age being a strong predictor of expressive vocabulary [29].

Pragmatic language (communication in social interactions) is delayed very early in development. Young children with FXS have been shown to have difficulty with joint attention, reciprocating positive facial expressions, eye gaze, and turn-taking [28, 32]. Deficits in pragmatic skills continue to surface as individuals develop. Studies indicate that males with FXS demonstrate high rates of tangential (off-topic) language, overly literal interpretation of language, as well as decreased topic initiation and maintenance [23]. Repetitive language (also called perseverative) is a hallmark of FXS and one that causes significant social difficulty.

Autism and FXS

About a half to two-thirds of males and about 20 % of females with FXS meet criteria for ASD [33]. These two disorders share multiple behaviors (Table 2), including pragmatic deficits, language delays, reduced eye contact, difficulty with regulation of attention and activity level, and additional problematic behaviors (e.g., self-injurious behaviors and aggression).

Characteristics that tend to differ between the FXS behavioral phenotype from the phenotype of idiopathic ASD in general include a higher rate of intellectual disability, more severe motor coordination deficits, worse expressive than receptive language, generally higher interest in socialization (although limited by anxiety), and better imitation skills in FXS relative to idiopathic ASD (Table 2).

Current Supportive Treatment of FXS

Current treatment of FXS is supportive with therapy, educational strategies that take into account cognitive and behavioral strengths and weaknesses in FXS, treatment of medical problems, behavioral modification, and psychopharmacology for behaviors creating dysfunction [33].

Given the global developmental delay most individuals with FXS display, it is likely that they will qualify for multiple services at a young age. Occupational therapy, physical therapy, and speech therapy should all be accessed at the maximum level allowed so as to maximize early growth. Therapy techniques that have been validated for autism are often helpful for FXS but must be modified based on what is known about the FXS phenotype.

Individuals with FXS have been shown to display better behavioral and adaptive functioning when their environment and supportive programming is well matched to their needs [37], and therefore a highly individualized behavioral, therapeutic, and educational intervention plan is needed both for home and school or work environments.

Psychopharmacologic treatment with medications such as stimulants for attention and hyperactivity, selective serotonin reuptake inhibitors for anxiety, alpha-agonists for hyperactivity and overarousal, and antipsychotics for irritable and aggressive behaviors appears to be helpful by assessment in a clinical setting in approximately 50–70 % of patients [34]. Response is not complete, however, and data from a national survey on FXS showed that approximately 10–20 % of respondents thought that medication was not helpful at all for behavior problems, whereas only approximately 40 % felt the medication was helping a lot [38]. Thus, there is a clear unmet

Table 2 Phenotypic differences between autism spectrum disorder (ASD) and fragile X syndrome (FXS)

	FXS (with and without ASD)	ASD
Cognition	• 90 % have IQs that qualify as an intellectual disability • Typically a decline in IQ with age in adolescence and early adulthood secondary to slowed development compared with typical peers	• <50 % qualify for an intellectual disability • IQ scores are relatively stable over time
Language and communication	• Expressive language worse than receptive • More perseverative language • Less impairment in social skills such as social smiling, joint attention, and engagement	• Receptive language worse than expressive • More echolalia • Greater impairment in social skills
Behavior	• Higher rates of anxiety • Higher rates of attention deficits/hyperactivity • Higher rates of perseverative behavior	• Higher rates of complex obsessive/compulsive behavior
Motor	• Significant delays in fine and gross motor skills	• Early motor development is typically unaffected, although mild motor difficulties may surface later

Information taken from Abbeduto et al. [35] and Ozonoff et al. [36]

need in FXS for better treatments for behavior and for any treatments that target cognitive deficits; thus, treatments that modify the underlying disorder would be an extremely important advance.

Molecular Functions of FMRP: Targets and Molecular Mechanisms

FMRP Domain Function and Human Missense Mutations Provide Insight into Canonical and Noncanonical Mechanisms

FMRP is an mRNA binding protein that regulates the translation of mRNAs encoding proteins that play important roles in neuronal development and synaptic function [39]. An understanding of molecular mechanisms of FMRP domains and target mRNA binding has provided important insight into mechanisms of translational control that go awry in FXS. These mechanisms have been reviewed extensively elsewhere [40]. Here, we will thus focus on recent discoveries and noncanonical mechanisms of FMRP-mediated regulation. FMRP contains three canonical RNA binding domains, two ribonucleoprotein K homology domains (KH1, KH2), and an arginine-glycine–glycine repeat-containing RGG box. An isoleucine 304 to asparagine missense mutation (Ile304Asn) in the KH2 domain, associated with severe clinical symptoms of FXS, has been shown to behave like a null mutation with reduced RNA binding and polyribosome association in a mouse model [41]. More recently, a patient diagnosed with FXS was found to have a glycine 266 to glutamic acid (Gly266Glu) missense mutation in the KH1 domain, which also disrupted RNA binding and polyribosome association [42]. Moreover, the Gly266Glu mutant was unable to rescue the excess α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization in *Fmr1* knockout (*Fmr1*^{KO}) neurons, which is a key cellular phenotype attributed to loss of translational control by FMRP [43]. The C-terminal RGG box has been shown to bind to G-quadruplex structures using multiple approaches [44–47], although the cellular function of this interaction is unknown. As G-quadruplex structures can facilitate mRNA localization to neuronal processes [48], and FMRP-target mRNAs have impaired activity-regulated mRNA localization in *Fmr1*^{KO} neurons [49], this suggests a possible role for the RGG box in mediating mRNA localization. Future work is needed to understand possible mechanistic inter-relationships between the RGG box and KH domains for regulation of FMRP-target mRNA translation.

The role of the amino terminus of FMRP in RNA binding has been reported in several studies, despite the lack of a well-conserved RNA binding domain [40]. Recent crystal structures of the amino terminus uncovered the existence of a novel KH0 domain (residues 126–202) [50]. Of potential interest, KH0 overlaps with the minimal domain (residues 180–217) required for FMRP binding to the small noncoding RNA, BC1 RNA [51]. The role of FMRP–BC1 interactions in translational regulation in neurons has been characterized [52, 53]. More work on the RNA targets and species recognized by KH0 may further clarify its role in mRNA regulation. The KH0 domain has recently been implicated in functions outside of FMRP's established role in regulating mRNA translation. Of interest, a patient with developmental delay, but not showing the clinical phenotype of FXS, has been identified with an R138Q missense mutation in KH0 [54]. This mutation does not impair canonical functions of FMRP involved in mRNA translation control but does impair presynaptic function and the protein–protein interaction between FMRP and BK channels [54].

FMRP contains nuclear localization and export sequences that facilitate nucleocytoplasmic shuttling [55]. FMRP binds mRNA in the nucleus [56], and has been shown to regulate RNA editing [57, 58]. Two FMRP isoforms (ISO6, ISO12) contain a unique C-terminus, which is absent in common isoforms, for example ISO1, ISO7, and contains a sequence that localizes FMRP to nuclear Cajal bodies [59]. A recent study has shown that the tandem Agenet (Tudor) domain mediates FMRP binding to chromatin involved in the DNA damage response [60]. It will be interesting to further explore possible noncanonical nuclear mechanisms of FMRP.

Mechanisms of FMRP-Mediated Regulation of mRNA Translation in Neurons

Perhaps the most extensively studied function for FMRP has been to repress mRNA translation [39]. Consistent with this role are numerous reports of excess mRNA translation and protein synthesis in animal models of FXS and cells from humans with FXS [61–66]. Recent studies suggest that FMRP-mediated repression involves its association with polyribosomes and ribosome stalling or direct interaction with the 80S ribosomal subunit [39, 67, 68]. Regardless of whether FMRP acts to stall ribosomes by binding indiscriminately along coding sequences [67] or directly to the ribosome [68], these models are unlikely to explain how FMRP selectively binds only 4 % of mRNA in brain [69]. The search for cis-elements is an important direction to pursue, and the role of short sequence motifs in FMRP targets has been reported. The identified motifs included a 7-nucleotide sequence containing a conserved GAC core in one study [70], and 2 shorter motifs ACUK (K = G/U) and WGGA (W = A/U) in another study [71]. Recent bioinformatics analysis of these 2 target mRNA data sets suggests enrichment of GACR and WGGA but not ACUK motifs in FMRP target mRNAs [72], the latter of which would contribute to G-quadruplex structures. Further work is needed to understand the role of cis-elements and secondary structures to confer translational regulation of FMRP target mRNAs. It will be interesting to see if different mRNA binding modalities of FMRP may act together to regulate mRNA translation of specific targets.

FMRP has also been shown to be a positive regulator of mRNA translation in cultured neurons and in the brain [73–76]. In these examples, mRNA targets were observed to have decreased polyribosome association and protein expression in *Fmr1*^{KO} mice. More work is needed to characterize the molecular mechanisms involved in the role of FMRP to stimulate translation.

For FMRP to play a key role in protein synthesis-dependent synaptic plasticity underlying learning and memory, it must be capable of reversible regulation of mRNA translation. Few studies have addressed the important question of how FMRP-mediated repression is removed. In one model, activation of group 1 metabotropic glutamate receptors results in the dissociation of cytoplasmic FMR1-interacting protein, a noncanonical eIF4E-BP, from the translationally repressed messenger ribonucleoprotein particle complex [53]. Post-translational modifications of FMRP play key roles in regulating translational repression. Multiple studies have shown that phosphorylation of FMRP at serine 499 in mouse (500 in human) results in translational repression [77–81]. Dephosphorylation of FMRP by PP2A in response to mGlu1/5 activation removes translational repression [82]. FMRP can also be ubiquitinated in response to mGlu1/5 activation [83], and this mechanism can occur locally within dendrites and at synapses [84]. These studies suggest that dephosphorylation and ubiquitination of FMRP are linked mechanistically to the removal of translational repression and receptor-induced protein synthesis.

FMRP Interactions with the microRNA Pathway

FMRP has been shown by several studies to associate with mature microRNAs (miRNAs) and Argonaute proteins, which are part of the RNA-induced silencing complex (RISC) [79, 85–89], and also to interact with other components of the miRNA pathway, including Dicer and miRNA precursors [90]. More recently, interactions between FMRP and RISC/miRNAs have been shown to regulate dendritic protein synthesis and spine morphology [79, 85]. It will be interesting if future studies show that FMRP interactions with G-quadruplexes provide a common mechanism to regulate miRNA seed site accessibility [91]. The tight association of phosphorylated FMRP with RISC/miRNAs may promote translational repression, whereas dephosphorylation of FMRP in response to activation of mGlu1/5 receptor results in release of miRISC and concomitant activation of translation [79]. A role for FMRP phosphorylation to regulate miRNA-mediated repression was recently observed in a non-neuronal culture system [92], which further supports the proposed model. FMRP may also play a role in miRNA stability or turnover, and a recent report suggests dysregulation of several miRNAs in the hippocampus from *Fmr1*^{KO} mice [93]. Of interest, FMRP was recently shown to have differential effects on mRNA translation, acting either positively or negatively, depending on the type of molecular interactions between FMRP and the RNA helicase, Mov10, with miRNA recognition elements and hairpin secondary structure [94]. Such interactions could result in either facilitation of RISC-mediated silencing or its inhibition. Taken together these studies suggest a mechanistic cooperation between FMRP and miRNAs that may influence translation in a bidirectional manner. Moreover, these studies suggest that dysregulation of miRNAs may underlie impairments in regulation of protein synthesis in FXS.

FMRP-mediated Regulation of Voltage-gated Ion Channels

Fmr1^{KO} neurons display increased neocortical network activity [95–98]. Recent evidence suggests that this hyperexcitability in the absence of FMRP may be caused by dysfunctions in voltage-gated ion channels [98, 99]. *In vitro* screening assays have identified several voltage-gated potassium, sodium, and calcium channels as potential FMRP mRNA targets [44, 67, 100, 101]. Two of them, the voltage-gated potassium channels Kv3.1b and Kv4.2, were validated in independent studies as FMRP mRNA targets [74, 102, 103]. While Kv3.1b was shown to be translationally repressed by FMRP, the exact modalities of FMRP-mediated regulation of Kv4.2 are not fully understood yet, and it is still controversial if FMRP acts as a translational suppressor or activator of Kv4.2 [74, 103]. Kv4.2 is the major mediator of A-type currents in the CA1 region of the hippocampus, and a recent study showed reduced A-type currents and increased backpropagating action potentials in CA1 pyramidal neurons in *Fmr1*^{KO} mice [104], suggesting that absence of FMRP decreases Kv4.2 function. Interestingly, apart from translational regulation, FMRP also directly binds to ion channels, which has revealed noncanonical functions. FMRP binds and activates Slack [105, 106], and FMRP's association with BK channels alters their calcium sensitivity in a protein synthesis-independent manner [98, 107]. A recent study also showed that FMRP interacts with the voltage-gated N-type calcium channel $Ca_V2.2$ and modulates its function and expression [108]. Taken together, these studies broaden our view of FMRP as a central regulator of ion channels suggesting novel routes of therapeutic strategies in FXS targeted at these channelopathies.

Preclinical Studies in the FXS Mouse Model

The underlying genetic cause of FXS, a trinucleotide expansion in the 5' untranslated region of *FMR1*, which leads to transcriptional silencing, is difficult to replicate in animal models. Instead, *Fmr1*^{KO} mice, in which a genetic deletion leads to complete lack of FMRP expression in all cells, are the predominantly used animal model for FXS [109, 110]. *Fmr1*^{KO} mice display FXS-like phenotypes (Table 3), and have been widely used to identify and assess potential therapeutic strategies (Table 4). Owing to space limitations we will not discuss the two invertebrate models of FXS, *Drosophila* and zebrafish [181–184], although these models, as far as they have been studied, show many of the same characteristics and rescue phenomena seen in the mouse model.

Recent Developments in the Characterization of *Fmr1*^{KO} Mice as a Preclinical Model for FXS

Fmr1^{KO} mice have been used to analyze the molecular functions of FMRP, to identify mechanisms underlying FXS and to test potential therapeutic strategies. Major efforts have been made to identify robust and reliable phenotypes in the mouse model that reflect the human disease and pathology and thus can be used to identify and test potential treatment strategies. These phenotypes range from molecular, cellular and network alterations to behavioral and cognitive deficits. However, many of these phenotypes are moderate, difficult to reproduce in different laboratories, and might thus not be reliable tools to evaluate potential therapeutic strategies in preclinical studies. The most frequently used and robust phenotypes are summarized in Table 3. Below, we will discuss recent developments in the phenotypic characterization of the FXS mouse model, which may enable more rigorous and physiologically relevant preclinical testing of therapeutic strategies in the future.

Molecular Defects as Potential Biomarkers

Fragile X is a disease of dysregulated signal transduction and lost control of general and target mRNA translation [39, 185]. Impairments in several signaling pathways and altered expression of many FMRP targets have been detected in the FXS mouse model. Recently, molecular defects that were originally observed in the brains of FXS animal models were also detected in peripheral blood lymphocytes, lymphoblastoid cell lines, and fibroblasts from patients with FXS. Thus, these molecular defects may serve as biomarkers to evaluate the efficiency of disease-targeting therapies in humans. Potential biomarkers for FXS include altered signaling activity of extracellular signal-regulated kinase (ERK)1/2 and phosphoinositide-3-kinase (PI3K) [64–66, 186–188], or altered protein expression or activity of FMRP targets [e.g., amyloid-β precursor protein (AβPP) or matrix metalloproteinase 9 (MMP-9) [162, 189]]. Not all defects in signaling in the mouse model have been reproducible across laboratories. In particular, reports about ERK1/2 and PI3K/mammalian target of rapamycin (mTOR) activation have been conflicting (e.g., [62, 63]). These discrepancies might be explainable by different tissues or tissue preparations used for analysis, but they also show that future work is needed to fully understand ERK1/2 and PI3K signaling defects in FXS in order to be able to comprehensively assess their potential as biomarkers in studies with humans. Nonetheless, impaired ERK1/2 activation and increased AβPP or MMP-9 levels have already been used as outcome measure in open-label clinical trials [189–192]. In the future, analyses of these biomarkers could help to assess if a specific drug treatment targets the underlying molecular mechanisms.

Synaptic, Neuronal, and Network Defects to Test Treatment Strategies

Phenotypes of animal disease models are considered particularly useful if they recapitulate a symptom in human patients. In the case of FXS, increased density and immature appearance of dendritic spines were detected in patients and mouse models [171, 193], and have been widely used to test therapeutic strategies. Pharmacologic and genetic modification of many molecular targets, including neurotransmitter receptors, intracellular signaling molecules and direct targets of FMRP have been shown to rescue this phenotype (Table 4), but it is unclear if correction of altered dendritic spine morphology in the mouse model is a good predictor of the success of a certain therapeutic strategy in patients [194]. More recently, Cruz-Martin et al. [195, 196] have identified defects in activity-induced dendritic spine dynamics in the FXS mouse model *in vitro* and *in vivo*, which may be a direct correlate of synaptic plasticity underlying learning and memory. Stimulus-insensitive dendritic spine dynamics and increased turnover of dendritic spines were also observed in two other reports [197, 198]. Of note, defects in dendritic spine dynamics were not rescued, but rather exaggerated, by chronic *in vivo* administration of the mGlu5 negative allosteric modulator (NAM) 2-methyl-6-(phenylethynyl) pyridine [195]. This is of special interest, because recent clinical trials with mGlu5 NAMs did not show significant behavioral improvements in adolescents and adults with FXS compared with placebo, although cognition and functional outcomes were not tested (Table 5) [194, 210]. In the future, it will be important to identify pharmacologic interventions that restore dynamic alterations in dendritic spine morphology in *Fmr1*^{KO} mice in response to extracellular stimuli, as those might be particularly promising therapeutic strategies for patients with FXS.

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Table 3 Major phenotypes of the fragile X syndrome mouse model used to assess therapeutic strategies

Category	Mechanism	Phenotype rescued	Correlate in patients
Molecular	**Protein synthesis**	Increased [62, 63, 111–113], stimulus-insensitive [62]	Lymphoblastoid cells [64], fibroblasts [66]
	AMPA receptor internalization	Increased [43, 62, 114]	No
	Altered cellular signaling	Increased [64, 111, 113] or decreased PI3K/mTOR phosphorylation [115]; increased ERK1/2 phosphorylation [112]; increased GSK3β phosphorylation [116]	Lymphoblastoid cells [64], lymphocytes and brain tissue [65], fibroblasts [66]
Cellular	**Dendritic spine density**	Increased (many studies; see Table 4)	Postmortem studies in brain tissue from patients [171]
	Dendritic spine morphology	Immature, fewer mushroom-shaped dendritic spines (many studies; see Table 4)	Postmortem studies in brain tissue from patients [171]
	LTD	Exaggerated , protein synthesis-independent [83, 172, 173]	No
Physiological	**Macro-orchidism**	Increased testes weight [111]	Macro-orchidism [5]
	Body weight	Increased body weight [111, 117]	Obesity in a subgroup of patients [174]
Neuronal hyperactivity	**Audiogenic seizures**	~40–80 % seizure susceptibility (many studies; see Table 4)	Increased susceptibility to epilepsy [175]
	PPI	Increased PPI (most studies, some show decreased PPI; see Table 4)	Impaired PPI [176]
	UP states	Prolonged [97, 150]	No
Behavioral	*Perseverative and repetitive behavior*	Increased or reduced marble burying, self-grooming [111, 162]*	(Yes) †
	Locomotor activity	Open field , rotarod [111]	Yes
	Anxiety	Open field, elevated plus maze, light–dark box‡	(Yes)†
	Social dominance, novelty and interactions	Social dominance (automated tube test [119]); social novelty and interactions [111, 132, 166]	(Yes)†
Cognitive	Short-term memory	Novel object recognition [111, 177]; fear conditioning [115]	(Yes)§
	Visual-spatial learning	Y-maze [111, 115], reversal Morris water maze, Hebb–Williams water maze [178]	(Yes)§

Shown in bold are phenotypes that have been used frequently (≥4 times) and showed consistent results. Phenotypes in regular font have been used less frequently but showed consistent defects. Phenotypes in italics were used to show rescue but the actual defects (e.g., increased *vs* reduced) varied in different studies.

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PI3K = phosphoinositide 3-kinase; mTOR = mammalian target of rapamycin; ERK = extracellular regulated kinase; GSK3β = glycogen synthase kinase 3β; LTD = long-term depression; PPI = prepulse inhibition

*Most studies show rescue of increased marble burying but at least 2 studies reported decreased burying [118, 162].

† These behaviors in rodents are often considered to recapitulate autistic-like behavior in humans. However, direct correlates are difficult to determine.

‡ Open-field behavioral analyses vary greatly, showing both hypo- and hyperanxiety [179].

§ Patients with fragile X syndrome are intellectually impaired but direct correlates to these memory tests in rodents are, again, difficult to determine. For a more extensive review of *Fmr1* knockout mice behavioral phenotypes see [180].

Table 4 Rescue strategies in *Fmr1* knockout mice

Target type	Specific target	Rescue type	Rescued/improved phenotypes	Phenotypes tested but not rescued
Neurotransmitter-dependent receptors	mGlu5	Genetic (het)	*Molecular and cellular*: protein synthesis, dendritic spine density, body weight [117] *Plasticity and hyperexcitability*: ocular dominance, AGS*, LTD [117] *Behavior*: social interactions [118]; partial rescue of social dominance [119] *Cognition*: inhibitory avoidance memory [117]	Macro-orchidism [117, 118]; locomotor activity, AGS*, PPI, startle response, OFA, MB [118]
		Pharm	*Molecular and cellular*: mTOR and ERK1/2 signaling [120, 121]; GSK3β phosphorylation [122, 123]; synaptic protein composition [124]; protein synthesis [62, 63, 120]; AMPA receptor internalization [43]; dendritic spine density (*in vitro* [125, 126]; *in vivo* [120, 127]); partial rescue of macro-orchidism [120] *Plasticity and hyperexcitability*: LTD (after *in vivo* administration) [120]; mEPSC frequency (amygdala) [128]; UP states [97]; OFA [121, 129, 130]; AGS [121, 130, 131], EPM [121] startle response [120],† PPI [125, 126]; *Behavior*: social dominance [119]; sociability [132] *Cognition*: object recognition (chronic, not acute) [121]; fear conditioning [120]; associative motor learning (acute) [133]; inhibitory avoidance (chronic and acute) [129, 133]; extinction memory (chronic) [129]	LTP deficits in the amygdala and hippocampus (bath application) [128, 134]; LTD (bath application) [135] startle response,† rotarod performance [131]; object recognition (acute) [121, 134]; coordinate and categorical tasks (acute) [134]; analgesic response [121] mGlu5 NAMs used: • MPEP [43, 62, 63, 97, 123, 126, 128, 130, 131, 134] • MTEP [121] • Fenobam [124, 126, 133] • AFQ056 [119, 125, 127, 132] CTEP [120, 129]
	mGlu1	Genetic (het)	*Hyperexcitability*: locomotor activity [118]	Macro-orchidism, PPI, startle response, AGS, social interaction [118]
		Pharm	*Hyperexcitability*: AGS (partially) [131]	Startle response, rotarod [131]
	Muscarinic R (subtypes M1 and M4)	Genetic (het)	*Plasticity and hyperexcitability:* analgesic response, startle response (M4) [136]	PPI, MB, light–dark transition (anxiety), AGS, macro-orchidism [136]
		Pharm	*Plasticity and hyperexcitability:* AGS (M1+M4) [137, 138] *Cognition*: passive avoidance (M4) [138]	Passive avoidance (M1) [137]
	Group II mGlu	Pharm	*Plasticity*: LTD [139]	N/A
	$GABA_A$ receptor agonist	Pharm	*Plasticity and hyperexcitability*: hyperexcitability in the amygdala [140]; AGS, EPM [141]; startle response [142] *Cognition*: fear conditioning [142]	PPI [142]
	$GABA_B$ receptor agonist	Pharm	*Molecular and cellular:* protein synthesis, AMPA receptor internalization, dendritic spine density [114] *Plasticity and hyperexcitability*: AGS [114, 143] *Behavior*: MB [114]	N/A
	Dopamine receptor agonist	Pharm	*Hyperexcitability*: hyperactivity (partial) (D1R agonist) [144] *Cognition*: object recognition (amphetamine) [145];	N/A
	5HT7 R agonist‡	Pharm	*Plasticity*: AMPA receptor internalization, LTD [146]	N/A
	TrkB agonist	Pharm		N/A TrkB agonists used:

Table 4 (continued)

Target type	Specific target	Rescue type	Rescued/improved phenotypes	Phenotypes tested but not rescued
			Plasticity and hyperexcitability: reduced TrkB phosphorylation [147]; hippocampal theta-burst LTP [148]; dendritic spine density (hippocampus and amygdala) [147] *Cognition*: Morris water maze, fear conditioning [147]	• BDNF [148] • 7,8-dihydroxyflavone [147]
	Cannabinoid receptor 1	Genetic (het)	*Molecular and cellular*: mTOR signaling [121] *Hyperexcitability*: analgesic response; AGS [121] *Cognition*: object recognition [121]	N/A
		Pharm	*Molecular and cellular*: mTOR signaling; dendritic spine density and morphology [121] *Hyperexcitability:* analgesic response; AGS [121] *Cognition*: object recognition (acute and chronic) [121][§]	OFA, EPM [121]
	Cannabinoid receptor 2	Pharm	*Plasticity and hyperexcitability:* OFA, EPM, AGS [121]	Object recognition (acute and chronic), analgesic response [121]
	NMDA receptor	Pharm	*Plasticity*: restores excitatory dendritic spine formation in cerebellar granule cells [149]	N/A
Scaffolding and cell signaling molecules	Homer 1a	Genetic (ko)	*Molecular and cellular*: DHPG-induced mTOR signaling, protein synthesis, mGlu5-Homer scaffolds [150] *Plasticity and hyperexcitability*: UP state duration, OFA, AGS (partial) [150]	LTD, increased expression of select FMRP targets (MAP1B, Arc, CaMKIIα) [150]
	RGS4	Genetic (ko)	*Molecular and cellular*: reduced forebrain PSD-95, $GABA_A$ receptor subunit α1, body weight [151] *Behavior*: social behavior (dominance and conditioned place preference) [151]	Macro-orchidism, OFA, novel object recognition [151]
	ERK1/2	Pharm	*Molecular and cellular*: protein synthesis in slices *Hyperexcitability*: AGS [63]	Activity-induced protein synthesis in synaptoneurosomes, AMPA receptor endocytosis [62]
	Ras (farnesylation)	Pharm	*Molecular and cellular*: protein synthesis [112] *Plasticity and hyperexcitability*: epileptiform discharges, cortical excitability, AGS [112]	N/A
	PI3K	Pharm	*Molecular and cellular*: DHPG-induced protein synthesis, AMPA receptor endocytosis [62], dendritic spine density and morphology [62, 152]	N/A
	GSK3β[¶]	Pharm	*Molecular and cellular*: GSK3β phosphorylation [122, 123, 153]; protein synthesis [113]; dendritic spine density (*in vivo*), body and testis weight (partial rescue) [153] *Plasticity and hyperexcitability*: LTP, reduced steady-state depolarization (dentate gyrus) [134]; LTD [139]; OFA [122, 123, 153, 154], EPM [123, 153, 154]; AGS [122][‖] *Behavior*: sociability [153, 155] *Cognition*: passive avoidance [123, 153]; object recognition coordinate and categorical tasks [134]	N/A GSK3β antagonists used: • lithium [113, 122, 123, 134, 153–155] specific GSK3β antagonists [122, 134]
	PAK	Genetic (dn)	*Cellular*: dendritic spine density (partially) [156]	N/A

Table 4 (continued)

Target type	Specific target	Rescue type	Rescued/improved phenotypes	Phenotypes tested but not rescued
		Pharm	*Plasticity and hyperexcitability*: LTD; OFA (partially) [156] *Cognition*: fear conditioning (partially) [156] *Cellular*: dendritic spine density [157] *Hyperexcitability*: OFA, AGS [157] *Behavior*: repetitive movements [157]	N/A
	PDE-4	Pharm	*Plasticity*: LTD (acute and chronic) [158]	N/A
	PTPase (PTEN)	Pharm	*Plasticity*: dendritic spine dynamics, LTP [159] *Cognition*: Morris water reversal learning [159]	The drug BpV is a general PTPase inhibitor with an inhibitory effect on PTEN
Regulators of translation	mTOR	Pharm	*Molecular and cellular*: protein synthesis in synaptoneurosomes (partial) [62], *Plasticity and hyperexcitability*: OFA, EPM [121]; AGS [121] (only partially [63]) *Cognition*: object recognition [121]	Protein synthesis in slices [63]; analgesic response [121] mTOR inhibitors used: • rapamycin [62, 63] • temsirolimus [121]
	TSC2	Genetic (het)	*Plasticity and hyperexcitability*: LTD [160] *Cognition*: fear conditioning [160]	N/A
	S6K	Genetic (ko)	*Molecular and cellular*: mTOR and ERK1/2 signaling, PSD-95, CaMKIIα, Shank3, eEF2, eIF4G protein levels, protein synthesis, dendritic spine density and morphology, body weight, macro-orchidism [111] *Plasticity and hyperexcitability:* LTD [111] *Behavior*: rotarod [111] *Cognition*: object recognition, Y maze [111]	OFA, MB [111]
	CPEB	Genetic (ko)	*Molecular and cellular*: S6 phosphorylation, expression levels of FMRP targets, protein synthesis, translation elongation rate, body weight [161] *Plasticity and hyperexcitability*: exaggerated LTD (but still protein synthesis-independent), glycine LTP, synapse formation, dendritic spine density, AGS (partially) [161] *Behavior*: nest building [161] *Cognition*: working memory (T maze) [161]**	N/A
Specific targets of FMRP	AβPP	Genetic (het)	*Molecular and cellular*: dendritic protrusion length (partially) [162] *Plasticity and hyperexcitability*: LTD, AGS (partially) [162] *Behavior*: OFA, MB [162]	N/A
	MMP-9	Genetic (ko)	*Molecular and cellular*: PI3K/mTOR signaling, dendritic spine morphology (*in vitro* and *in vivo*), macro-orchidism [163] *Plasticity and hyperexcitability*: LTD, OFA [163]	N/A
		Pharm††	*Molecular and cellular:* reduces MMP-9 levels, dendritic spine morphology (*in vitro* and *in vivo*) [164] *Hyperexcitability*: EPM [164] *Cognition*: Y maze [164]	N/A
	p110β	Pharm	*Molecular and cellular*: protein synthesis [64]‡‡	N/A
		Genetic (het)	*Molecular and cellular:* DHPG-induced protein synthesis, p110β- and mGlu5-associated PI3K activity, dendritic spine density [237]	UP states [237]

Table 4 (continued)

Target type	Specific target	Rescue type	Rescued/improved phenotypes	Phenotypes tested but not rescued
			Hyperexcitability: AGS [237] *Behavior:* MB, nest building [237] *Cognition*: goal-directed behavior, behavioral flexibility [237]	
	PIKE	Genetic (het)	*Molecular and cellular:* DHPG-induced protein synthesis, p110β- and mGlu5-associated PI3K activity, DHPG-induced PI3K activity, dendritic spine density [238] *Plasticity and Hyperexcitability*: LTD, AGS, UP states [238] *Behavior:* MB, nest building [238]	N/A
	STEP	Genetic (het + ko)	*Plasticity and hyperexcitability:* EPM (only STEP het) AGS (partially), light–dark box (anxiety) (partially) [165] *Behavior*: social approach (STEP heterozygous only), social dominance (STEP ko only) [165]	N/A
	BK_{CA}	Pharm	*Molecular and cellular*: brain glutamate homeostasis, dendritic spine morphology (*in vitro*) [166] *Hyperexcitability:* EPM (partially), dendritic excitability, sensory hypersensitivity [98] *Behavior*: sociability, social novelty [166] *Cognition:* Y maze [166]	N/A
	Kv4.2[§§]	Pharm	*Plasticity:* LTP [103]	N/A

Shown are genetic and pharmacological rescue strategies and their effects in *Fmr1* knockout mice. The following strategies were confirmed in studies using *Drosophila* models of fragile X syndrome (FXS): metabotropic glutamate receptor (mGluR) [167], gamma-aminobutyric acid (GABA) [168] (but see [169]), lithium [167], minocycline [170], phosphodiesterase-4 (PDE-4) [158]

AßPP = amyloid ß precursor protein; AGS = audiogenic seizure; AMPA = a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF = brain-derived neurotrophic factor; BpV = dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate; CaMKIIa = calcium/calmodulin-dependent protein kinase II alpha; CPEB = cytoplasmic polyadenylation element binding protein; CTEP = 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine; EPM = elevated plus maze; DHPG = 3,5-dihydroxyphenylglycine; dn = dominant-negative; eEF2 = eukaryotic translation elongation factor 2; eIF4G =eukaryotic translation initiation factor 4G; ERK = extracellular signal-regulated kinase; FMRP = fragile X mental retardation protein; GSK3ß = glycogen synthase kinase 3ß; het = heterozygous; ko = knockout; LTD = long-term depression; LTP = long-term potentiation; MAP1B = microtubule-associated protein 1B; MB = marble burying; mEPSC = miniature excitatory postsynaptic currents; MMP-9 = matrix metalloproteinase 9; MPEP = 2-methyl-6-(phenylethynyl) pyridine; MTEP = 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine; mTOR = mammalian target of rapamycin; N/A = not applicable; NAM = negative allosteric modulator; NMDA = N-methyl-D-aspartate; OFA = open field activity; pharm = pharmacological; PAK = p21-activating kinase; PI3K = phosphoinositide -3-kinase; PIKE = phosphoinositide 3-kinase enhancer; PPI = prepulse inhibition; PSD-95 = postsynaptic density protein-95; PTPase = protein tyrosine phosphatase; PTEN = phosphatase and tensin homolog; STEP = striatal-enriched protein tyrosine phosphatase; TrkB = tropomyosin receptor kinase B; TSC2 = tuberous sclerosus complex 2.

*The protocols used for AGS induction were slightly different but still do not fully explain these contradictory results.

† Two different mGlu5 NAMs were used for these studies.

‡ A recent study suggested that simultaneous stimulation of D1R and serotonin (5HT) receptors rescued impaired synaptic AMPA receptor insertion and plasticity, and improved fear conditioning and Y maze learning deficits [115].

§ Simultaneous inhibition of mGlu5 and CB1R was additive in this study.

¶ GSK3ß mRNA was identified as potential mRNA binding to FMRP in a screen but has not been validated in independent studies as an FMRP target [67].

¦ Effect of simultaneous inhibition of mGlu5 and GSK3ß inhibition on AGS was not additive in this study.

**Working memory was also rescued by acute depletion of CPEB in the hippocampus.

†† Minocycline activates MMP-9 but also has other effects.

‡‡ Increased protein synthesis is also rescued in FXS patient cell lines [64, 66].

§§ The drug used (HpTx2) blocks all Kv4 channels.

Table 5 Clinical trials of targeted treatments in fragile X syndrome (FXS)

Drug	Target	Design	Phase	Length*	Age (years)	$n^{\dagger}$	Outcomes measured		Efficacy outcome
							Primary	Other	
CX516 [199]	3	DB, PC	II	4 wks	18+	48	Memory z-score (TVPS, W-JMem, RBANS)	CGI-I, VAS, VABS, executive z-score (IVA, SNAPIV), behavior z-score (ABC, CARS, GARS, ADOS), language z-score (CELF, PLS-4, PPVT)	None in full group, CGI better CX516 *vs* placebo in antipsychotic-treated group
Lithium [200]	1B	OL	IIa	2 mo	6–45	15	ABC-I	ABC-T, ABC, VAS, CGI-I, RBANS-LL/SM, PPVT, CFXCPT, NEPSY Tower, NVALT, Card Task, VABS, ERK, eye tracking, RSA, HR, HRV, auditory filter	ABC-I (primary) not improved, ABC-T and 3 subscales, VAS, CGI, RBANS, and ERK improved
Fenobam [201]	1A	OL	IIa	1 d	18+	12	Safety	PPI, CFXCPT	PPI improved over test–retest controls
Donepezil [202]	4	OL	I	6 wks	14+	8	CNT	HVLT, ABC, CBCL	CNT, ABC-T, ABC-I, ABC-H, CBCL improved
Donepezil [203]	4	DB, PC	II	12 wks	12–29	45	ABC-T	MRS, working memory tests	In progress
Minocycline [204]	2	OL	IIa	2 mo	13–35	20	ABC-I	ABC, CGI-I, VAS, RBANS, PPVT, NVALT, VABS, SB-IV	ABC-T, ABC, CGI-I, VAS improved
Minocycline [205]	2	DB, PC, XO	II	3 mo	3.5–16.0	55	CGI-I, VAS	VAS—multiple behaviors, ABC, EVT, VABS, MMP-9 activity in blood	CGI-I , some VAS and MMP-9 improved over placebo, small effect size
AFQ056 [206]	1A	DB, PC, XO	II	4 wks	18–45	30	ABC-T	ABC subscales, RBS, CGI-I, CGI-S, VAS, SRS, KiTAP, RBANS–LL/SM, PPVT, ERK, eye tracking, PPI	ABC-T, ABC, CGI, RBS, CGI-I, VAS, SRS improved in fully methylated group
AFQ056 [203]	1A	DB, PC	IIb	3 mo	18–45	162	ABC-T	ABC, CGI-I, CGI-S, RBS, SRS, KiTAP/eye tracking (some sites—not analyzed yet)	No improvement on behavioral scales over placebo
AFQ056 [203]	1A	DB, PC	IIb	3 mo	12–17	140	ABC-T	ABC, CGI-I, CGI-S, RBS, SRS, TEA-Ch, KiTAP/ eye tracking (some site—not analyzed yet)	No improvement on behavioral scales over placebo
AFQ056 [203]	1A	OL	I	1 wk	3–11	20	Safety, PK	ABC, TEA-Ch	N/A
RO4917523 [203]	1A	DB, PC	IIa	6 wks	18–50	40	Safety, PK	ABC, ADAMS, CGI-I, CGI-S, VAS, RBANS-LL/SM, PPI, KiTAP, eye tracking	Results not in public domain
RO4917523 [203]	1A	DB, PC	IIb	3 mo	14–40	185	ADAMS-Total score	ABC, SRS, CGI-I, CGI-S, ADAMS, GBAS, VAS, KiTAP RBANS-LL/SM, eye tracking	Did not meet primary outcome, results not in public domain
RO4917523 [203]	1A	DB, PC	IIa	3 mo	5–13	47	Safety	PK, ABC, ADAMS, CGI-I, CGI-S, VAS, RBANS-LL/SM	Results not in public domain
Arbaclofen [207]	4	DB, PC, XO	II	4 wks	6–50	62	ABC –I	ABC, VAS, CGI-I, CGI-S, CASI, ADHDRS, RBS, SRS, SB5-WM, RBANS, PPVT, VABS, APP	ABC-I not improved over placebo, ABCFX-SA, VAS improved over placebo, ABS-SW, ABCFX-SA, CGI, VAS, VABS-P/L all improved over placebo in low social group
Arbaclofen [208]	4	DB, PC	III	8 wks	12–50	125	ABCFX-SA	ABCFX, CGI-I, CGI-S, VAS, PSI, SRS, VABS	Did not meet primary outcome

Table 5 (continued)

Drug	Target	Design	Phase	Length*	Age (years)	$n^{\dagger}$	Outcomes measured		Efficacy outcome
							Primary	Other	
Arbaclofen [208]	4	DB, PC	III	8 wks	5–11	172	ABCFX-SA	ABCFX, CGI-I, CGI-S, VAS, PSI, SRS, VABS	Full data not analyzed yet
Acamprosate [209]	4	OL	II	10 wks	5–17	12	CGI-I	ABC, SRS, CASI, ADHDRS, PPVT, VABS, APP, BDNF	CGI-I, ABC-SW, ABC-H, ADHDRS, SRS improved
Acamprosate [203]	4	DB, PC	II	10 wks	5–23	48	ABC-SW	CGI-I, CGI-S, VAS, other ABC, ADAMS, SRS, PedsQL, CSQ, CYBOCS, VABS, PARS, ELS, eye tracking, ERK, APP	In progress
Ganaxolone [203]	4	DB, PC, XO	II	6 wks	6–17	60	CGI-I	VAS, ABCFX, ADAMS, PARS, SNAPIV, KiTAP, PPI, eye tracking, ERP	In progress
NNZ-2256 [203]	1B	DB, PC	II	6 wks	14–40	60	Safety	CGI-I, CGI-S, ABCFX, SRS, CASI, VABS, VAS-P, VAS-C, ELS, PARS, FXS rating scale, CYBOCS, KiTAP, eye tracking, ERK	In progress
Metadoxine [203]	1B, 4	DB, PC	II	6 wks	14–50	60	ADHDRS	ABCFX, CGI-I, CGI-S, SRS, KiTAP, PARS, VABS, eye tracking	In progress

ABC = Aberrant Behavior Checklist-Community Edition—all 5 individual subscales (ABC-H = Hyperactivity Subscale; ABC-I = Irritability Subscale; ABC-SW = Social Withdrawal Subscale; ABC-T = Total scale score); ABCFX = Aberrant Behavior Checklist-Community Edition refactored for FXS—all 6 individual subscales (ABCFX-SA = Social Avoidance Subscale); ADAMS = Anxiety Depression and Mood Scale; ADHDRS = ADHD Rating Scale IV; ADOS = Autism Diagnostic Observation Scale; APP = amyloid precursor protein; BDNF = brain-derived neurotrophic factor; Card Task = Card Task Test of Visual Sequential Memory; CARS = Childhood Autism Rating Scale; CASI = Childhood Anxiety Sensitivity Index; CBCL = Child Behavior Checklist; CELF = Clinical Evaluation of Language Skills; CFXCPT = Carolina Project Fragile X Continuous Performance Test; CGI-I = Clinician Global Impression-Improvement; CGI-S = Clinician Global Impression-Severity; CNT = Contingency Naming Task; CSQ = Caregiver Strain Questionnaire; CYBOCS = Children's Yale–Brown Obsessive Compulsive Scale modified for pervasive developmental disorders; DB = double-blind; mo = months; ELS = expressive language sampling; ERK = extracellular regulated kinase-related kinase activation rate; ERP = evoked response potentials; EVT = expectancy-value theory; GARS = Gillam Autism Rating Scale; GBAS = Global Behavior Assessment Scale; HR = heart rate; HRV = heart rate variability; HVLT = Hopkins Verbal Learning Test; IVA = Integrated Visual-Auditory Continuous Performance Test; KiTAP = Tests of Attentional Performance for Children; MMP-9 = matrix metalloproteinase-9; MRS = magnetic resonance spectroscopy; N/A = not applicable; NEPSY = developmental NEuroPSYchological assessment; NVALT = Non-Verbal Associative Learning Task; OL = open label; PARS = Pediatric Anxiety Rating Scale; PC = placebo-controlled; PedsQL = Pediatric Quality of Life Scale; PK = pharmacokinetics; PLS-4 = Preschool Language Scale – Version 4; PPI = prepulse inhibition; PPVT = Peabody Picture Vocabulary Test; PSI = Parenting Stress Index; RBANS (LL/SM) = Repeatable Battery for the Assessment of Neurological Status (LL = List Learning, SM = Story Memory); RBS = Repetitive Behavior Scale; RSA = Respiratory Sinus Arrhythmia; SB5-WM = Stanford–Binet Version 5 Working Memory Index; SB-IV = Stanford–Binet intelligence scale edition IV; SNAPIV = Swanson, Nolan, and Pelham Questionaire for Attention Deficit Hyperactivity Disorder; SRS = Social Responsiveness Scale; TEA-Ch = Test of Every Day Attention for Children; TVPS = Test of Visual Perceptual Skills; W-JMem = Woodcock–Johnson Memory for Words; VABS = Vineland Adaptive Behavior Scale (P/L = Play and Leisure Subscale); VAS = visual analog scale (VAS-C = clinician rated; VAS-P = parent rated); wk(s) = week(s); XO = crossover;

*Length of treatment period analyzed in trial

† Number of subjects in trial

intracellular signaling molecules and direct targets of FMRP have been shown to rescue this phenotype (Table 4), but it is unclear if correction of altered dendritic spine morphology in the mouse model is a good predictor of the success of a certain therapeutic strategy in patients [194]. More recently, Cruz-Martin et al. [195, 196] have identified defects in activity-induced dendritic spine dynamics in the FXS mouse model *in vitro* and *in vivo*, which may be a direct correlate of synaptic plasticity underlying learning and memory. Stimulus-insensitive dendritic spine dynamics and increased turnover of dendritic spines were also observed in two other reports [197, 198]. Of note, defects in dendritic spine dynamics were not rescued, but rather exaggerated, by chronic *in vivo* administration of the mGlu5 negative allosteric modulator (NAM) 2-methyl-6-(phenylethynyl) pyridine [195]. This is of special interest, because recent clinical trials with mGlu5 NAMs did not show significant behavioral improvements in adolescents and adults with FXS compared with placebo, although cognition and functional outcomes were not tested (Table 5) [194, 210]. In the future, it will be important to identify pharmacologic interventions that restore dynamic alterations in dendritic spine morphology in *Fmr1*^{KO} mice in response to extracellular stimuli, as those might be particularly promising therapeutic strategies for patients with FXS.

A prominent defect in synaptic plasticity in *Fmr1*^{KO} mice is exaggerated and protein synthesis-independent metabotropic glutamate receptor-dependent long-term depression [83, 172]. However, this synaptic deficiency is difficult to translate into a specific defect in patients, and thus might not be ideal to test therapeutic strategies. In contrast, patients with FXS show symptoms of neuronal hyperexcitability, such as increased susceptibility to seizures and hyperarousal. This neuronal hyperexcitability may be reflected by a heightened susceptibility to audiogenic seizures in FXS mice [211], a widely used phenotype to assess therapeutic strategies in preclinical analyses. More recent efforts have focused on identifying measurable defects in neuronal network activity in *Fmr1*^{KO} mice that can be used to test therapeutic strategies. For example, prolonged neocortical UP states, a network defect in FXS that reflects the overall neuronal hyperexcitability [95, 96], have been successfully decreased with genetic or pharmacological reduction of mGlu5, genetic removal of Homer 1A, genetic reduction of the PI3K enhancer (PIKE), but not p110β [97, 150, 237, 238]. Of note, a recent study has identified a potential neuronal correlate of hyperarousal in the somatosensory cortex of *Fmr1*^{KO} mice that was rescued pharmacologically using a BK_{CA} channel opener [98]. The same BK_{CA} activator has been used previously to rescue behavioral impairments in the FXS mouse model [166], suggesting that neuronal network hyperexcitability is a useful phenotype to evaluate therapeutic strategies in FXS.

Assessing Activity-Dependent Neuronal Defects to Test Therapeutic Strategies in FXS

Many molecular and cellular phenotypes in *Fmr1*^{KO} mice are subtle, difficult to reproduce in different laboratories and often discussed controversially. These problems most likely arise from differences in genetic background, handling, and housing of the mice, which may alter the steady-state levels of intracellular signaling, protein synthesis, and dendritic spine morphology. A prominent feature of FMRP-deficient cells is the impairment in stimulus-induced neuronal responses. This has been shown on the molecular level (protein synthesis and signal transduction; e.g., [62, 63]), the cellular level (dendritic spine dynamics [196, 198]), and more recently on the network level (hypersensitivity of neocortical responses to tactile stimuli [98]). Exploring defects of stimulus sensitivity in animal models of FXS as phenotypes to test therapeutic strategies is particularly attractive because of their relevance for synaptic plasticity underlying learning, memory, and behavior. Moreover, these defects may be observed regardless of the "baseline" constitutive phenotype. Of note, impairments in stimulus-induced ERK and Akt signaling and protein synthesis have also been shown in fresh blood lymphocytes and lymphoblastoid cell lines from patients with FXS [64, 187].

Behavioral and Cognitive Phenotypes in the FXS Mouse Model

Behavioral analyses of *Fmr1*^{KO} mice are mainly focused on autism-associated defects, for example perseverative behavior, anxiety, and impairments in social interaction (see Table 3; reviewed in [180]). Behavioral phenotypes of FXS mice vary considerably across laboratories, and even defects in the opposite directions have been reported frequently [179]. To improve comparability of results, a future challenge will be to establish rigorous protocols and standards for behavioral testing in FXS that can be applied in various laboratories. Moreover, it will be important to identify more robust and reliable behavioral defects. Here, recent advances in analyzing social behavior and social interactions in FXS mice may hold some promise [119, 161, 212].

FXS is an intellectual disability, and the ultimate goal for the development of therapeutic strategies is to improve cognitive function in patients. Initial analyses to assess defects in hippocampal dependent learning and memory using classic Morris water maze assays revealed only subtle and varying defects in *Fmr1*^{KO} mice [213]. Recently, more robust defects in novel object recognition were reported by several laboratories [111, 145, 214], making this paradigm a promising tool to test therapeutic strategies for the improvement of cognition in FXS. Of note, patients with FXS are mostly impaired in higher cognition, behavioral flexibility, and inhibitory control, which depend on the prefrontal cortex. To date, very few studies have reported defects in prefrontal cortex-dependent higher-order cognition in *Fmr1*^{KO} mice [215, 237]. A recent study showed that defects in higher cognition in FXS mouse models were rescued by genetic reduction of the PI3K catalytic subunit p110β in the prefrontal cortex [237]. An important future direction will be to use defects in higher cognition in FXS mouse models to assess the efficacy of pharmacological rescue strategies in improving intellectual capabilities in FXS.

Therapeutic Targets and Rescue Strategies in the FXS Mouse Model

Rescue strategies in the FXS mouse model have been based on the analysis of molecular pathomechanisms (e.g., dysregulated mGlu1/5 signaling and protein synthesis), identification of specific FMRP targets that are dysregulated in FXS (e.g., increased protein levels of MMP-9 or striatal-enriched protein tyrosine phosphatase), or have been guided by observations in human patients, and the availability of Food and Drug Administration-approved drugs (*N*-methyl-D-aspartate antagonists [149]; lovastatin [112]).

Rescue strategies that were tested in the FXS mouse model target virtually every step of cellular signal transmission: neurotransmitter receptors, scaffolding proteins that connect receptors with downstream signaling, intracellular signaling molecules, and regulators of protein synthesis. In some cases, the pharmacologically or genetically modified molecules were confirmed FMRP targets, making these rescue strategies particularly promising. A summary of rescue strategies tested in the mouse model is shown in Table 4. In the following sections, we will discuss a few emerging themes and challenges arising from these studies.

Neurotransmitter Receptors Versus Intracellular Signaling and mRNA Translation

Significant effort has focused on manipulating neurotransmitter-dependent receptors as a therapeutic strategy in FXS. The most widely studied rescue approach targets dysregulated mGlu1/5 signaling in FXS using mGlu5 NAMs [216], which were shown to rescue many phenotypes in both mouse and fly models of FXS. Clinical trials using this approach have, so far, been unsuccessful in showing an improvement of FXS-associated phenotypes in humans, possibly because only behavioral phenotypes were addressed in older patients [194]. Of note, recent studies in the mouse model showed that chronic treatment with mGlu5 NAMs is necessary to improve memory deficits [120, 121, 129], suggesting that prolonged treatment in patients may also be beneficial. Notably, targeting mGlu5 seems to be more efficient in rescuing phenotypes in the FXS mouse model than targeting other G-coupled receptors that were also shown to be dysregulated in FXS [217], such as mGlu1 or muscarinic receptors M1 and M4 [118, 131, 136–138].

Another strategy has been to increase the signaling activity of certain receptors that may have reduced function in FXS, such as gamma-aminobutyric acid (GABA), dopamine and serotonin receptors, or tropomyosin receptor kinase B (Table 4). Here, agonists to GABA and to dopamine receptors are specifically promising because signaling through them was shown to be altered in FXS animal models [144, 169].

In general, neurotransmitter receptors are preferred pharmacological targets; however, in the case of FXS their therapeutic potential maybe limited. A hallmark of FXS animal models and patient cells is the lack of stimulus-induced intracellular signaling and protein synthesis. Dampening the upstream receptor may successfully reduce basal activity but may not restore stimulus sensitivity. Instead, recent studies suggest that strategies targeted at intracellular molecules may be more efficient in restoring the stimulus-induced response of signal transduction and protein synthesis [62, 159]. Extensive efforts have been made to identify signaling molecules or receptor scaffolding molecules that play important roles in the FXS phenotype. Genetic deletion of scaffolding molecules downstream of mGlu1/5 (Homer1a) or general G-coupled proteins (RGS4) have shown rescue of select phenotypes [150, 218]; however, so far it is not clear how they can be used as a pharmacological target in FXS. Furthermore, broad spectrum inhibitors of ERK1/2 and PI3K showed improvement of a few phenotypes in FXS mice but, owing to expected side effects, will not be suitable for treatment in humans [62, 63, 152]. Other efforts have focused on selective phenotypes of FXS, such as, for example, dendritic spine morphology by targeting p21-activating kinase [156, 157], or altered cyclic adenomonophosphate levels in patients with FXS by targeting phosphodiesterase-4 [158, 219].

The absence of FMRP leads to dysregulated protein synthesis, and a general protein synthesis inhibitor was shown to rescue phenotypes in a fly model of FXS [220]. In line with this observation, genetic or pharmacological reduction of central molecules involved in the regulation of general mRNA translation (mTOR [121], S6K1 [111], tuberous sclerosis 2 [160]) and molecules involved in the mRNA translation of select targets (cytoplasmic Poly(A)-binding protein [161]) have been used successfully in the mouse model to rescue many phenotypes. Future studies will have to show if general reduction of mRNA translation is a safe therapeutic strategy in humans.

Manipulating Specific Targets of FMRP

Other approaches have pharmacologically or genetically manipulated select targets of FMRP, such as AβPP, striatal-enriched protein tyrosine phosphatase, MMP-9, and the BK_{CA} channel (Table 4). These strategies led to reversal of several phenotypes in the mouse model. In the future, it will be interesting to further analyze how targeting one out of many

FMRP-regulated proteins with restricted and specialized functions in the cell can be therapeutic in FXS. Of particular interest will be strategies that modify FMRP targets with known central functions in intracellular signaling, such as the PI3K enhancer (PIKE), the PI3K catalytic subunit p110β or the glycogen synthase kinase-3β (GSK3β) [62, 122, 155, 221, 237, 238]. Both signaling molecules were identified in recent screens for FMRP targets [67, 71], and p110β has been validated in independent studies as an FMRP target [62, 221]. Moreover, lithium, an antagonist of GSK3β, is already approved for use in humans, and p110β-selective inhibitors have been developed for cancer research. While mouse studies with lithium and a specific GSK3β antagonist already show promising effects on behavior and cognition in FXS mice [122, 123, 134, 155], so far, p110β-selective inhibitors have only been validated *in vitro* in mice and *in vivo* in patient-derived cell lines [64, 66].

Challenges and Future Outlook for Preclinical Studies

As outlined above, an extensive number of pharmacologic and genetic rescue strategies have been tested in *Fmr1*^{KO} mice and most of them show rescue of a wide range of phenotypes. However, several of these studies also report that specific defects in the mouse model are not rescued by the tested strategy (Table 4). The comparison of defects that were and were not rescued in these different approaches may help to clarify underlying molecular mechanisms, evaluate the potential efficiency to improve core deficits in human patients, and, lastly, suggest potential combinatory therapies using drugs targeting different pathways. Currently, such analyses are complicated by the fact that virtually none of the studies has tested (and/or reported) the same battery of phenotypes, making a direct comparison nearly impossible. Future studies evaluating the effect of different drug treatments in side-by-side experiments will be instrumental to further assess therapeutic strategies.

The identification and development of potential treatment strategies in FXS has been driven substantially by the use of *Fmr1*^{KO} mice. These have been a valuable tool to delineate molecular mechanisms underlying FXS and to reveal the cellular functions of FMRP. However, a major problem for preclinical studies in FXS has been that most phenotypes in the mouse model are rather subtle and difficult to reproduce in different laboratories. So, while many different genetic or pharmacological rescue strategies have shown improvements in these phenotypes (see Table 4), the overall effects have been small owing to the subtle nature of the analyzed neuronal and behavioral deficits. This might have contributed to the disappointing results of recent clinical trials that were based on these studies (see following sections) [194]. Thus, a better comprehension of the benefits and limitations of the FXS mouse model is necessary to be able to utilize it as a reliable preclinical tool. One study, for example, revealed differences in autistic-like behaviors of *Fmr1*^{KO} mice depending on the genetic background, and argued that some phenotypes might rather represent the specific strain than the absence of FMRP expression [222]. In the future, it will be essential to better understand how genetic background affects phenotypes in the FXS mouse.

Recently, an *Fmr1*^{KO} rat model was generated, but, so far, very few studies using this new animal model have been published. One study showed altered speech representation in the auditory cortex of *Fmr1*^{KO} rats [223], and another study discovered some autistic-like behaviors but no defects in basic memory tasks [224]. As rats are generally the preferred model to test more complex cognitive function, *Fmr1*^{KO} rats hold great promise to evaluate therapeutic strategies for improving intellectual impairments and deficits in higher cognition in FXS.

Another promising novel tool for preclinical studies of brain disorders are human neurons that were generated from patient-derived induced pluripotent stem cells (iPSCs). The recent advances in the use of iPSCs derived from human cells enable the testing of therapeutic strategies "in a dish" [225]. Early studies in FXS patient-derived iPSCs suggest that some FXS-associated defects are replicated in these cells [226–228], although the classic phenotypes reported in the mouse model, for example exaggerated protein synthesis and altered dendritic spine morphology, have yet to be examined in FXS iPSC-derived neurons. In the future, it will be important to identify robust phenotypes in human iPSC-derived neurons from FXS that are suitable to test or screen for therapeutic drugs.

Translation of Targeted Treatments to Humans with FXS

Based on the work with FXS models discussed in the previous sections, treatment targets have been identified for potential clinical development and directed at 1) reduction of excess activity in signal transduction pathways connecting group 1 metabolic glutamate receptors (mGluRs) or other Gq-linked receptors to the dendritic translational machinery, either through (1A) receptor modulation at the cell surface or (1B) through modulation of the intracellular signaling pathway; 2) reduction of excessive activity of proteins normally regulated by FMRP; 3) increasing expression and activation of surface AMPA receptors; 4) modification of activity of GABA and other receptors/proteins that regulate glutamate signaling or translational signaling pathways; 5) using miRNAs to block excessive translation of mRNAs normally regulated by FMRP; and 6) correction of abnormal channel activities normally directly regulated by FMRP (see Table 5, which reviews all targeted treatment trials in FXS with ≥ 8 participants) [229, 230].

Successful preclinical testing in FXS models has led to early proof-of concept clinical trials and subsequent larger trials for some of the proposed targeted treatments (Table 5). CX516, an AMPA activator, did not show efficacy but was used most likely at a subtherapeutic dose, a conclusion that would be supported by a suggestion of efficacy in patients co-treated with antipsychotics, known to potentiate effects of CX516 [199]. Lithium, thought to reduce excess mGluR-dependent activation of translation by attenuating GSK3β activity and possibly phosphatidyl-inositol turnover, resulted in significant improvement in behavioral scales, verbal memory, and abnormal ERK phosphorylation rates in lymphocytes in a 2-month pilot open-label proof-of-concept trial in children and young adults with FXS [200]. A pilot placebo-controlled crossover trial of minocycline, an antibiotic that inhibits overexpressed synaptic MMP-9 in FXS models, conducted in children with FXS demonstrated mild global clinical improvement and reduction of MMP-9 levels in the blood of responders [205].

GABA-B agonist arbaclofen presumably lowers presynaptic glutamate release with resultant reduction of group 1 mGluR signaling (Table 5). In a phase II double-blind placebo-controlled crossover trial [207], arbaclofen showed improvement over placebo in the entire per-protocol group for social withdrawal and parent-nominated problem behaviors. However, a large phase III placebo-controlled trial in adolescents and adults with FXS did not show benefits for arbaclofen over placebo in the primary outcome of social withdrawal. An additional phase III trial in children with FXS showed promise but full analyses are pending. Acamprosate, currently Food and Drug Administration-approved for alcohol withdrawal, with agonist properties at both GABA-A and GABA-B receptors, has shown promise, in an open-label trial, for hyperactivity and social functioning in FXS [209]. Acamprosate and the GABA-A agonist ganaxolone are being tested in small placebo-controlled trials in FXS [203].

Multiple negative modulators of the mGluR5 receptor have been in trials in FXS (Table 5). A single oral dose of fenobam resulted in a significant improvement in abnormal prepulse inhibition compared with untreated control participants with FXS [201]. A phase II double-blind, placebo-controlled, crossover trial of AFQ056 in 30 men with FXS treated for 28 days each with AFQ and placebo suggested improvement in maladaptive behavior in a post hoc analysis in the subgroup with full methylation of *FMR1* [206]. Larger multinational trials of AFQ056 and RO4917523, another mGluR5 negative modulator (Roche), have not supported this behavioral outcome but have not addressed cognitive or learning outcomes [203]. Clinical observations from long-term extension studies with both arbaclofen and AFQ056 have suggested there may be long-term cognitive and functional benefits of these drugs that were not captured by formal measures employed in the trials [231].

Challenges and Future Outlook for Clinical Studies

Although many neuronal targets for treating the underlying disorder in FXS have been identified, translational work has begun, and, as can be seen in Table 5, early open-label and some phase II trials have suggested benefits for multiple targeted treatments, there have been problems with demonstrating disease modification in subsequent larger trials because there are still many uncertainties about how to demonstrate treatment effects optimally in a clinical trial setting [229, 232, 233]. Major trial design issues in FXS trials potentially include variable but narrow dosing windows, timing (age) and length of treatment necessary, potential need for cognitive or behavioral interventions to see drug effects on synaptic plasticity in the form of learning, large placebo effects, difficulty predicting a primary outcome in a disease such as FXS with diverse cognitive and behavioral manifestations for a drug that reverses phenotypes in animal models, and lack of validated, sensitive biomarkers, and functional outcome measures in FXS [232]. These trial design issues will need to be resolved to be able to demonstrate disease modification in FXS. It has been recognized that more objective clinician-administered and direct observational measures, measures that address cognition and are validated for populations with intellectual disability, and biomarkers of central nervous system function are needed to reduce placebo effect and establish that the drug is "hitting the target", and these measures are being increasingly incorporated into more recent trials (Table 5). It is likely that efficacy studies will need to focus on younger study populations, and intensive learning interventions will need to be employed within the trial design in the drug and placebo groups in order to see the enhancement of synaptic plasticity seen in animal models within a time frame compatible with placebo-controlled trials. Thus, the overall approach to process of drug development in FXS needs to change such that efficacy is no longer required in adults before studying children, ways of analyzing global outcomes over multiple domains are developed and accepted by regulatory authorities, and good precedents are established and accepted for embedding form of cognitive training within trial designs. If these problems can be solved, hopefully, in the future, treatment to reverse the underlying disorder will eventually replace or complement supportive treatment.

There is significant overlap in molecular and cellular pathways involving FMRP and those including gene products associated with ASD [6, 230]. This overlap falls roughly into 3 categories: 1) defects in proteins linked to the signaling cascade for regulation of FMRP-regulated translation such as Shank, mTOR, p21-activating kinase, and phosphatase and tensin homolog; 2) defects in proteins regulated directly by FMRP such as postsynaptic density protein 95 and Arc; and 3) defects in proteins involved in the balance of activity in brain glutamate and GABA systems. Indeed, the FMRP/ASD pathway overlap has been recently supported by the findings that 1) in a meta-analysis of exome screening studies, FMRP binds to about a third of all genes associated with ASD [234]; 2) genes that code for FMRP target mRNAs are more likely than other genes with similar expression patterns to contribute to ASD [235]; and 3) common variants in genes involved in postsynaptic regulation of FMRP activity (CAMK4, GRM1, cytoplasmic FMR1-interacting protein) are risk factors for ASD [236]. Treatments directed at all of these pathway-overlap areas are being explored in preclinical or clinical translational work in FXS and, if successful, progress in development of such targeted treatments for FXS may result in treatments to reverse neural defects and clinical manifestations of ASD and intellectual disability.

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