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Phase II and III drugs for the treatment of fragile X syndrome

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Introduction: Fragile X syndrome (FXS), the most common inherited form of intellectual disability and autism, is caused by expansion of CGG trinucleotide repeats in *FMR1* and marked reduction or absence of the gene product, FMRP. FMRP suppresses synaptic protein synthesis resulting from group 1 metabotropic glutamate receptor (mGluR) activation, a process critical to normal synaptic plasticity. FXS can be characterized as a disorder of synaptic plasticity with physical, cognitive and behavioral manifestations attributable to, at least in part, excessive mGluR activity and downstream effects.

Areas covered: This paper reviews the 'mGluR theory' of FXS and the targeted drugs investigated in Phase II and III trials based on this theory of pathogenesis. A literature review was conducted using the PubMed database with search terms 'fragile X syndrome', 'mGluRs', 'pharmacotherapy' and specific drug-related terms. Other resources were identified by review of relevant reference lists and consultation with experts in the field.

Expert opinion: While preclinical trials of targeted drugs in animal models of FXS have been encouraging, more studies are needed to determine clinical efficacy in humans. Challenges to clinical trial design and direction for future drug studies, including consideration of NMDA receptor partial agonists and mGluR2/3 agonists, are discussed.

Keywords: autism, FMRP, fragile X syndrome, GABA, GABA receptor agonists, glutamate, group 1 metabotropic glutamate receptors, intellectual disability, mGluR2/3 agonists, mGluR5 antagonists, NMDAR partial agonists, oxytocin, synaptic plasticity

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1. Introduction

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability (ID) and autism with approximately 1/2500 – 4000 individuals affected by the full mutation [1-3]. The genetic mutation resulting in the fragile X phenotype was first identified in 1991 on the long arm of the X chromosome at Xq27.3, and the affected gene was designated fragile X mental retardation-1 (*FMR1*) [4]. FXS results from the unstable expansion of CGG trinucleotide repeats in the 5′ untranslated region of *FMR1*, leading to hypermethylation of the promoter region and silencing of *FMR1* transcription [4]. The full mutation resulting in FXS contains > 200 CGG repeats and is associated with the absence or markedly reduced levels of the gene product, fragile X mental retardation protein (FMRP), an mRNA binding protein [5-7] that regulates translation of proteins important in synaptic plasticity [8-10]. Smaller amplifications, termed premutations (55 – 200 CGG repeats), do not result in the absence of FMRP but rather increased levels of *FMR1* mRNA, which is purported to have a direct toxic effect in cells [11-14]. Mosaicism occurs in 20 – 40% of males, indicating the presence of the full mutation in some cells and premutation in others [15]. The number of trinucleotide repeats frequently undergoes expansion in the offspring of female carriers, with the risk of expansion to full mutation directly proportional to the number of carrier CGG repeats [15].

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Article highlights.

- Fragile X syndrome (FXS) is a single-gene disorder with characteristic cognitive, physical and behavioral features that overlap many more common neurodevelopmental disorders, including intellectual disability (ID) and autism spectrum disorder (ASD), and serves as an important neurobiological model for developing targeted drug treatments.
- The proposed disease mechanism in FXS involves overactivity of group 1 metabotropic glutamate receptors (mGluRs) and increased synaptic protein synthesis in the absence of translational inhibition normally provided by Fragile X Mental Retardation Protein-1 (FMRP), leading to impairments in long-term depression (LTD) and synaptic plasticity.
- Modulators of glutamatergic and GABAergic neurotransmission have the potential to ameliorate behavioral symptoms and possibly improve cognitive deficits in FXS.
- Newly developed *N*-methyl-D-aspartate receptor (NMDAR) partial agonists and mGluR2/3 agonists are drugs that have not been studied in FXS to date and warrant further investigation.

This box summarizes key points contained in the article.

Individuals with the full mutation exhibit a broad array of cognitive, emotional and behavioral impairments, including mild to severe ID, learning disability, hypersensitivity to sensory stimuli, attention deficit hyperactivity disorder (ADHD), anxiety, social phobia, agitation, aggression, self-injury and sleep disturbance [15,16]. Approximately 20 – 30% of individuals with FXS also meet criteria for autistic disorder [17], and many more have features of autism, including repetitive and stereotyped behaviors, poor eye contact and social impairment [18]. The severity of autistic features appears to increase with lower levels of FMRP and is stable over time [17]. ADHD symptoms, particularly hyperactivity, are observed in approximately 75% of younger boys with FXS and typically decline with age [15,18]. Agitation and aggression also follow a developmental course, peaking in early adolescence around the time of puberty and fading for most by young adulthood [18]. The rate of anxiety disorders exceeds 75% in individuals with the full mutation, substantially greater than in other populations with ID [19]. Anxiety is typically manifested as poor eye contact, gaze aversion and excessive shyness but may also drive other common behaviors, including hand flapping, self-injury, aggression and autistic mannerisms [18]. In a study of 97 subjects with FXS, 58% met *Diagnostic and Statistical Manual-IV* (DSM-IV) criteria for social phobia, 59% for specific phobia and 25% for selective mutism, a particularly rare and severe form of social anxiety [19].

Characteristic physical features in FXS include a large head circumference, long, narrow face, prominent forehead, closely set eyes, large ears, highly arched palate, joint hyperextensibility and macroorchidism [15,16,20]. Seizures are common, affecting 13 – 20% of males and 5% of females with the full mutation [15,16]. Seizures most often occur in childhood, typically resolving before adulthood [16].

In general, the severity of cognitive disability and physical phenotype is inversely proportional to the amount of FMRP produced [21,22]. Autistic behaviors are also more prominent among individuals with lower levels of FMRP [17]. Males with the full mutation typically have mild to moderate ID, with those demonstrating a mosaic pattern less severely affected than those with a fully methylated pattern [15]. Females with FXS are typically less severely affected than males due to higher levels of FMRP production associated with the presence of one normal X chromosome [16]. Approximately 50 – 71% of females with the full mutation have an IQ in the borderline to mild mental retardation range [15,23,24], though others have subtle learning difficulties and no apparent physical manifestations of the syndrome. The degree of clinical expression in females depends in part on the ratio of X chromosome inactivation (XCI) in cells [15,24]. In females who inherit the full mutation and have skewed inactivation of the normal allele, the cognitive, physical and behavioral phenotype is more severe [24,25].

Premutation carriers (55 – 200 CGG repeats) usually have intellectual abilities in the normal range, though they may exhibit mild cognitive and behavioral difficulties, including social anxiety, learning disabilities and occasionally ID and autism [14,26]. As with individuals with the full mutation, the degree of intellectual impairment in male carriers depends on the amount of expressed FMRP [21]. Both males and females with premutations exhibit particular difficulty with arithmetic, suggesting an underlying mechanism for specific cognitive deficits other than FMRP depletion in the premutation state [21,22]. Excess *FMR1* mRNA has been identified in male premutation carriers, particularly in those with 100 – 200 CGG repeats, and is likely due to increased rates of transcription as a result of reduced translational efficiency [11,21,22].

Carrier status is also associated with disorders of adult-onset, primarily premature ovarian failure (POF) in women and Fragile X Tremor- Ataxia Syndrome (FXTAS) in men [14,16,27]. Approximately 16% of women with fragile X premutations exhibit POF, which is unrelated to CGG repeat size or deficient FMRP [14,27]. Unlike with the full mutation, skewing of XCI is not associated with the development of POF [28]. FXTAS, developing in 30 – 40% of male carriers in their 50s and beyond, includes intention tremor, gait ataxia, parkinsonism, autonomic dysfunction, executive function deficits and memory loss with progression to dementia in some individuals [14,16,29,30]. The vast majority of carriers who develop late-onset cerebellar ataxia have larger ($\geq$ 70) CGG repeat sizes, however, and the actual prevalence of this syndrome in the general population is likely lower than originally reported [31]. Neuroimaging reveals characteristic white matter lesions in the middle cerebellar peduncles, brain atrophy and periventricular white matter disease [12,14].

Eosinophilic intranuclear inclusions, identified on autopsy throughout the cerebrum and brainstem in a group of premutation carriers, are ubiquitin-positive, suggesting probable intranuclear accumulation of proteins as in other gain-of-function trinucleotide expansion diseases [13,14]. Female premutation carriers develop neurological symptoms much less commonly, an observation that exceeds what would be expected due to X-inactivation effects and suggests additional gender-specific factors [14].

2. Target symptom management

In clinical practice, pharmacotherapy for FXS has historically focused on alleviating the associated emotional and behavioral manifestations of the disorder [32]. A parent survey of more than 1300 children with FXS found that 61% of males and 38% of females were taking medication for at least one symptom, most commonly anxiety and inattention, though parents considered most medications only somewhat effective [32]. Well-designed pharmacological studies for FXS are sparse, and FXS is typically an exclusion criterion in larger trials of individuals with autism [33].

Based on the observation that leukocytes grown in medium deficient in folic acid reveals a fragile site at Xq27.3, several studies have investigated the effects of folic acid treatment in FXS. Though an initial open-label trial and some case reports suggested clinical benefit, no subsequent placebo-controlled trials have shown statistically significant improvement in cognitive or behavioral symptoms with folate treatment [34-40]. A randomized, placebo-controlled, crossover study of 21 males with FXS treated with the metabolically active form of folate, folinic acid, also failed to demonstrate statistically significant benefit on measures of adaptive behaviors, language skills and hyperactivity [41]. However, two subjects withdrew during the crossover arm of the study due to loss of substantial behavioral improvements seen during the first treatment arm; parents had correctly guessed treatment with folinic acid in both cases. A 1996 Cochrane Database review of folic acid in FXS, including review of the five controlled trials between 1986 and 1992, concluded that the studies lacked sufficient power to detect anything but large effects and that no definitive conclusions could be drawn from available data [42]. No actual deficits in folate metabolism have been demonstrated in animal models or humans with FXS, and efforts to investigate folate as a treatment have been abandoned [18].

ADHD symptoms are a common target for pharmacological intervention in FXS, though controlled trials of stimulants and non-stimulant medications are sparse [32]. In 1988, Hagerman *et al.* reported improvement in hyperactivity and social skills in 10 of 15 children treated with methylphenidate as compared with placebo and dextroamphetamine [43]. An observational study of 12 boys (mean age, 8 years 5 months) with FXS taking either methlyphenidate or dextroamphetamine suggested a response rate of 75% for improved attention, a rate greater than that typically observed in children with autism spectrum disorders (ASD) and idiopathic ID [44]. Academic performance was also enhanced in 75% of subjects during achievement tests with stimulant treatment, though no improvements were seen in excessive motor activity. Of note, the subjects in this small sample were not evaluated for *DSM-IV* diagnosis of ADHD, and no subjects were scored in the 'clinically significant' range on the DSM-ADHD rating scale at study entry.

L-Acetylcarnitine (LAC) has been studied as a treatment for hyperactivity in FXS based on the finding that the carnitine ester, involved in *N*-acteylation of histones at *FMR1*, caused modest reduction in *FMR1* promoter hypermethylation in two of three FXS lymphoblastoid cell lines [45-47]. Decrease in *FMR1* methylation status was not associated with transcription reactivation *in vitro* [45]. In a double-blind, placebo-controlled trial of 63 boys with FXS, LAC was well-tolerated and associated with significant reductions in hyperactivity on the Conners' Global Index for Parents, though not the Conners' Teacher form [47]. Vineland Socialization and Adaptive Behavior ratings also improved significantly for the LAC group compared with placebo; cognitive abilities remained unchanged. Quantitative polymerase chain reaction (PCR) did not detect *FMR1* mRNA after 12 months of treatment with LAC, suggesting that observed effects were likely due to its effect on carnitine metabolism.

Similarly, Torrioli *et al.* reported on valproate, a histone deacetylase inhibitor, as a treatment for ADHD symptoms in a 6-month, open-label study of 10 boys with FXS, finding significant improvement in hyperactivity scores on the Conner's Parent Rating Scale-Revised Short Form [48]. Two subjects withdrew from the study prematurely due to worsened behavior and restlessness.

Melatonin was effective in a randomized, double-blind, placebo-controlled crossover study for decreasing sleep-onset latency and modestly increasing mean night sleep duration in a sample of 12 children with FXS and/or autism [49].

A 12-week, open-label pilot study of aripiprazole in 12 children and young adults with FXS demonstrated significant improvement in irritability, including tantrums, aggression and self-injury, in 10 of 12 (87%) subjects [33]. Adverse effects were generally mild and included drooling, tiredness and insomnia.

3. mGluR theory of FXS

Following the discovery of the genetic defect leading to the fragile X phenotype, a knockout (KO) mouse model was designed to characterize the structure and function of FMRP [50]. Interruption of the *Fmr1* gene, the murine homolog to *FMR1*, resulted in mice lacking *Fmr1* protein and exhibiting macroorchidism, mild spatial learning deficits and increased motor activity [50]. KO mice also demonstrated an increased propensity for audiogenic seizures and long, thin, immature dendrites on autopsy, similar to humans with

FXS and other forms of ID [51-55]. *Drosophila* with mutation of the *FMR1* homolog *dfmr1* also demonstrated dendrites with abnormal synaptic branching and overgrowth [56]. The immature appearance and overgrowth of dendritic spines in humans and animal models suggested altered synaptic plasticity [54] and impairment in the 'pruning' of extraneous synaptic connections [57].

FMRP has been identified as a widely expressed RNA-binding protein with five functional domains, including two K homology (KH) domains and one RGG box that bind mRNA [5,6]. FMRP preferentially binds via the RGG box to mRNA containing a G-quartet (Gq) structure [10,53,58]. The majority of FMRP is found in the cytoplasm associated with polyribosomes and is likely involved in repressing translation of target mRNAs via this association [59]. Polyribosome-free FMRP, multiple mRNAs and proteins also aggregate to form translationally dormant messenger ribonucleic proteins (mRNPs) [7,60] that associate with cytoskeletal microtubules for transportation to neuronal dendrites [59]. On activation of excitatory, postsynaptic group 1 metabotropic glutamate receptors (mGluRs), translation rapidly ensues, due in part to dissociation of FMRP from target mRNA [61]. One of many consequences of the resultant protein synthesis is long-term depression (LTD) and a reduction in synaptic transmission [62]. If unchecked, excessive LTD can lead to synapse elimination [52,63]. When mGluRs are activated by presynaptic glutamate release, FMRP is also synthesized and inhibits further protein synthesis, creating a negative feedback mechanism for limiting mGluR-dependent LTD [60].

Appropriate inhibition of further protein synthesis by FMRP leads to reversion of synaptic changes within 30 min [52]. In the absence of FMRP, as in FXS, translational inhibition is lifted, and protein synthesis-dependent LTD is exaggerated, thus altering synaptic plasticity [52,64].

More than 500 target mRNAs have been identified [57,65], encoding proteins that themselves suppress translation of synaptic proteins [52], maintain proper neuronal function and mediate neuronal and craniofacial development [10,58,66]. One target protein of interest, for example, is microtubule-associated protein 1B (MAP1B), a protein involved in synapse development [56,67]. MAP1B homologs are overexpressed in mouse and *Drosophila* KO mutants and are associated with increased neuronal microtubule stability and synaptic overgrowth and branching, respectively [56,67]. These morphological changes in animal models are analogous to the long, thin, immature dendritic spines in individuals with FXS and suggest that upregulated MAP1B may play a role in impaired synapse development and possible cognitive deficits in FXS [67]. *Fmr1* KOs also overexpress amyloid precursor protein (APP) and its proteolytic product, Aβ, implicated in the increased susceptibility to seizures observed in FXS, Alzheimer's disease and Down's syndrome [61]. While FMRP is well characterized as an mRNA-binding protein, Brown *et al.* recently demonstrated that FMRP also directly binds and activates the membrane protein Slack, a sodium-activated potassium channel involved in neuronal firing patterns [68].

Synaptic plasticity is determined by patterns of long-term potentiation (LTP) and LTD, the synaptic processes implicated in learning and memory [52]. While LTP promotes strengthening of synapses, LTD is an activity-dependent synaptic weakening important in synaptic pruning [52]. *Fmr1* KO mice show markedly reduced LTP in the cortex and amygdala [69], as well as significantly increased LTD in the hippocampus [70], the latter dependent on activation of group 1 mGluRs [53,70].

mGluR activation and subsequent protein synthesis ultimately leads to internalization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, the ionotropic glutamate receptors, from the postsynaptic membrane [63,71]. Removal of excitatory glutamate receptors from the synapse is one proposed mechanism for the expression of mGluR-dependent LTD [63]. AMPAR trafficking at the synaptic membrane plays a role in plasticity, and excessive internalization of AMPARs in the absence of FMRP may underlie impairments in learning and memory seen in FXS [71]. The GluR1 subunits of AMPARs in postsynaptic membranes of amygdala neurons are reduced in *Fmr1* KO mice, suggesting that impaired AMPAR expression could be the downstream manifestation of dysregulated LTP [69]. Alternatively, dysregulated LTP could well be the manifestation of impaired AMPAR expression, possibly due to excess mGluR5 signaling during early development. FMRP regulates the translation of proteins STEP and Arc, which are likely responsible for AMPAR endocytosis in LTD [57]. Aberrant internalization of AMPARs is rescued in cultured hippocampal neurons treated with the mGluR5 antagonist 2-methyl-6-phenylethynyl pyridine (MPEP), though not in amygdala neurons [69,71]. The failure of mGluR5 antagonism to normalize AMPAR expression in the amygdala does not necessarily indicate a mechanism for impaired amygdala LTP distinct from impaired hippocampal LTD, however. Dysregulated LTP in the amygdala may be a consequence of overall reduced synaptic connectivity in this region resulting from increased mGluR5-dependent protein synthesis during the development of amygdala circuitry.

Messenger RNAs for several gamma-aminobutyric acid-A ($GABA_A$) receptor subunits in fragile X mouse and *Drosophila* models are also underexpressed in association with FMRP deficiency [72]. The mechanism by which FMRP regulates $GABA_AR$ expression is not fully understood but may involve transport and localization of $GABA_AR$ mRNA [72]. $GABA_ARs$ are the primary inhibitory receptors in the brain, and decreased GABAergic activity in the cortex could contribute to symptoms of anxiety, depression, insomnia and epilepsy, as well as disturbances in learning and memory seen in fragile X patients [18,72]. Despite reduced $GABA_AR$ activity, *Fmr1* KO mice remain sensitive to the sedative and anxiolytic effects of $GABA_AR$ agonists, and the audiogenic seizure phenotype is corrected by treatment with the $GABA_AR$ agonists diazepam

and ganoxolone. These findings provide the rationale for targeting $GABA_AR$ activity in therapeutic drug trials [73].

Group 1 mGluRs consist of mGluR1, primarily expressed in the cerebellum, and mGluR5, expressed in the neocortex, hippocampus and striatum [65]. mGluR5 is of particular interest in FXS given its activity in the neocortex and hippocampus [65]. The group 1 mGluRs are G-protein coupled and transduce excitatory signals via three signaling cascades: activation of phosphoinositol (PI) hydrolysis by phospholipase C (PLC); the phosphoinositide (PI3K)/AKT/mammalian target of rapamycin (mTOR) cascade and the extracellular signal-regulated kinase (ERK) cascade [65]. All three pathways are involved in the regulation of protein synthesis and act together to initiate mRNA translation [65]. FMRP inhibits translation of many of the proteins participating in the signaling cascades, and in *Fmr1* KO mice, signaling proteins are overexpressed, further driving protein synthesis [57].

Based on the findings that i) mGluR activation results in protein synthesis and ii) protein synthesis is exaggerated in the absence of FMRP, Bear *et al.* proposed the 'mGluR theory' of FXS: that the varied functional consequences of mGluR-dependent protein synthesis can account for the cognitive, behavioral and physical phenotypes that characterize the syndrome [52,74]. Exaggerated LTD in FXS would favor synaptic loss during critical periods of synaptogenesis, accounting for developmental delay and impaired cognitive development [52]. *FMR1* is widely expressed throughout the brain, including in most cells with postsynaptic mGluRs, and overactivation of group 1 mGluRs in various regions could explain fragile X symptoms as varied as anxiety, seizures, loose bowel movements, hyperalgesia, obsessive–compulsive symptoms, poor coordination, tactile hypersensitivity and disturbed sleep patterns [52]. Antagonists of group 1 mGluRs reduce protein synthesis triggered by mGluR activation, and it follows that mGluR antagonists could potentially alleviate impairments in the FXS phenotype [52].

The mGluR theory of fragile X is supported by the effects of the non-competitive mGluR5 antagonist MPEP in the treatment of *Drosophila* KO mutants, rescuing short-term memory, abnormal courtship behavior and mushroom body defects [75,76]. Courtship impairment, indicative of learning and memory deficits, was corrected throughout development and into adulthood, though anatomical defects in the mushroom body, a structure analogous to the mammalian brain, were only rescued if treated early in development [75]. MPEP also suppresses the increased propensity toward seizures and excessive motor activity observed in *Fmr1* KO mice [61,77], though chronic mGluR5 inhibition has a smaller effect on macroorchidism. These findings suggest that aspects of the FXS behavioral phenotype may improve with mGluR5 antagonism throughout development, though some anatomical differences are less amenable to correction unless treatment occurs during a critical window in development.

Further support for the mGluR theory comes from the study of a double mutant mouse model with both *Fmr1* KO and 50% genetic reduction in mGluR5 expression [78]. Heterozygote crosses showed reduction in audiogenic seizures and normalization of the prepubescent accelerated growth and abnormal dendritic spine density observed in *Fmr1* KO mice. Altered ocular dominance plasticity (ODP) in the visual cortex and increased protein synthesis in the hippocampus of *Fmr1* KOs were also rescued by reduced mGluR5 expression. Testicular enlargement was not affected by genetic reduction of mGluR5. FMRP and mGluR were thus observed to act in functional opposition, with FMRP acting as a down-regulator of synaptic mRNA translation and protein synthesis triggered by mGluR5 activation [65,78]. In the absence of FMRP regulation, unchecked mGluR-dependent protein synthesis results in expression of the fragile X phenotype [65].

Validation of the mGluR theory suggests that medications targeting mGluR5, as well as components of the associated signaling cascades, could be therapeutic in individuals with FXS [79]. Downstream molecules regulated by the mGluR pathway, including individual proteins, GABARs, AMPARs and NMDARs, could also be targets for pharmacological intervention (**Figure 1**). While no medications specifically modulating the mGluR pathway have approval from the Food and Drug Administration (FDA) for treatment in FXS, a number of Phase II and III trials have been completed or are underway, as will be described below (**Table 1**).

4. mGluR5 antagonists

Extracellular antagonists of group 1 mGluRs, particularly mGluR5, have garnered much excitement for therapeutic potential based on the finding that 50% knockdown of mGluR5 activity in *Fmr1* KO mice and mGluR5 blockade with MPEP in animal models can correct aspects of the fragile X cognitive and behavioral phenotype, including propensity toward seizures, increased motor activity, memory impairment, repetitive behavior, sensorimotor processing impairment and accelerated prepubescent growth [61,75,77,78,80,81]. MPEP has also been shown to correct deficits in presynaptic glutamate release in the amygdala of *Fmr1* KO mice, suggesting a possible therapeutic role for symptoms of anxiety and hyperarousal in FXS [69]. Antagonists of mGluR5 have been identified as better therapeutic targets than mGluR1 antagonists, as mGluR1 is widely expressed in the cerebellum, and blockade is associated with motor impairment [57,80]. mGluR1 antagonists also appear less potent than mGluR5 antagonists in altering the fragile X phenotype [80]. In a recent study, the non-competitive mGluR1 antagonist JNJ16259685 diminished marble burying, a measure of repetitive behavior, in *Fmr1* KO mice but failed to significantly affect motor activity, motor coordination or susceptibility to seizures, as MPEP did [80]. MPEP is the prototypical mGluR5 antagonist but is not specific for mGluR5 and also inhibits NMDARs at high concentrations, leading to significant side effects [82]. Consequently, MPEP has not been studied in human subjects as attention has turned to more selective mGluR5 antagonists.

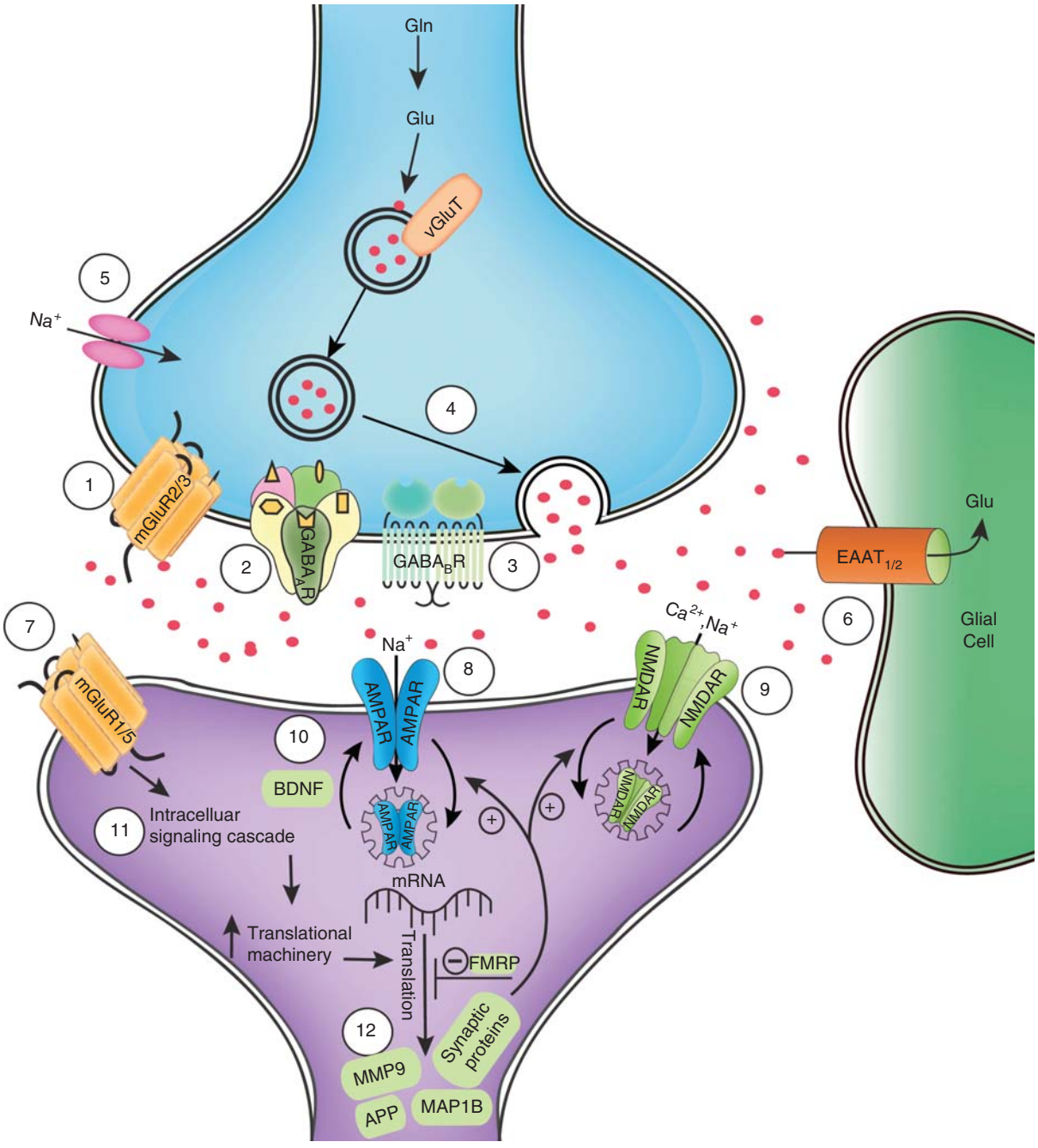

Figure 1. Illustration of potential targets for drug therapy at a glutamatergic synapse in FXS. When stimulated by presynaptic glutamate release, postsynaptic mGluR1/5 receptors activate intracellular signaling cascades that mobilize translational machinery to translate mRNA into synaptic proteins, examples of which include MMP-9, MAP1B and APP. FMRP inhibits translation of more than 500 target mRNAs in the normal state; in FXS, this negative feedback mechanism is absent or severely reduced, leading to excessive protein synthesis and altered synaptic plasticity. Consequences of increased synaptic protein synthesis include loss of AMPARs and NMDARs from the postsynaptic membrane via endocytosis. Drugs that modulate excitatory glutamatergic and inhibitory GABAergic neurotransmission have the potential to alleviate some phenotypic effects in FXS. Key for labeled mechanisms of action and corresponding drugs: 1) LY2140023 (mGluR2/3 agonist); 2) acamprosate ($GABA_AR$ agonist); 3) arbaclofen ($GABA_BR$ agonist); 4) riluzole (blocks presynaptic glutamate release); 5) riluzole (blocks voltage-dependent sodium channels); 6) riluzole (enhances glutamate reuptake); 7) MPEP, fenobam, AFQ056, RO4917523, STX107, CTEP, acamprosate (mGluR5 antagonists); 8) CX516 (ampakine); 9) memantine (weak NMDA antagonist); D-serine, D-cycloserine, GLYX-13 (glycine site NMDAR partial agonists); 10) lithium, CX516 (increase BDNF); 11) lithium (GSK3 inhibition); 12) minocycline (MMP-9 inhibition).

Table 1. Comparison of Phase II and III clinical trials in FXS.

Compound	Mechanism of action	Clinical findings
Fenobam	mGluR5 antagonist	Open-label, single-dose trial in 12 adults: 6/12 (50%) with > 20% improvement in PPI; 9/12 (75%) with observed 'calmed' behavior; no effect on attention and response inhibition [85]
AFQ056	mGluR5 antagonist	Randomized, double-blind crossover study in 30 male adults: No improvement on ABC-C subscales in subjects with partial *FMR1* methylation; signification improvement in ABC-C sum score and Stereotypy, Hyperactivity and Inappropriate Speech subscales in those with full methylation; fatigue and headache common [87] Phase II trials in adults and adolescents and Phase I trials in children underway [86-89]
Acamprosate	Probable mGluR5 antagonist, $GABA_AR$ agonist and weak NMDA antagonist	Open-label study in 3 adult males: subjective improvement in expressive language skills; no improvement in non-verbal communication [94] Phase III trial in children underway [95]
RO4917523	mGluR5 antagonist	Phase II trials in adult males underway [96]
STX107	mGluR5 antagonist	Phase II trials in adult males in development [97]
Riluzole	Exact mechanism unknown; believed to block presynaptic glutamate release, enhance glutamate reuptake, block voltage-dependent sodium channels	Open-label study in 6 adult males: 1/6 (16%) with reduction in repetitive behavior and irritability; decreased ERK activation in all patients; 5/6 (83%) with asymptomatic increase in LFTs [100]
Memantine	NMDA antagonist	Chart review of 6 young adults treated with memantine: 4/6 (67%) with improvement on CGI-I; no specific improvement in specific rating scales; 2/6 (33.3%) with treatment-emergent irritability [101]
Minocycline	MMP-9 inhibitor	Open-label study in 20 young adults: significant improvement in ABC-C Irritability subscale scores, CGI-I and VAS; 2/20 (10%) seroconverted to positive ANA; GI side effects common [106] Phase II trials in children recently completed; results not yet available [109]
Lithium	GSK3 inhibitor; increases BDNF production	Open-label study in 15 children and adults: no improvement in ABC-C irritability subscale scores; mild to moderate improvement in ABC-Hyperactivity and Inappropriate Speech subscale scores, CGI-I, VAS and VABS measures; 11/15 (73%) with polyuria/polydipsia; 4/15 (27%) with elevated TSH [111]
Arbaclofen	$GABA_BR$ agonist	Phase II randomized, placebo-controlled trial in 63 children and adults: trend toward significance in ABC-C Irritability subscale scores and CGI-I scores; 15 severely affected children with significant improvement in CGI-I and ABC-Social Withdrawal subscale scores [122] Phase III trials in children and adults underway [124]
CX516	Positive modulation of AMPA receptors	Randomized, placebo-controlled trials in 49 adults: no improvement on cognitive and behavioral measures [133]
OT	Neuropeptide involved in pro-social behavior	Randomized, placebo-controlled trial in 10 young adult males: Increased eye contact during social challenge task at lowest dose; decreased cortisol levels at highest dose [138]
Donepezil	Acetylcholinesterase antagonist	Open-label trial in 9 adults: improvement in measures of working memory, cognitive flexibility, hyperactivity, irritability, inattention [139] Randomized controlled trial in young adults underway [140]

ABC-C: Aberrant Behavior Checklist-Community Edition; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF: Brain-derived neurotrophic factor; CGI-I: Clinical Global Impressions-Improvement; FXS: Fragile X syndrome; GSK3: Glycogen synthase kinase-3; LFT: Liver function test; mGluR: Metabotropic glutamate receptor; MMP-9: Matrix metalloproteinase-9; NMDA: *N*-methyl-D-aspartate; OT: Oxytocin; PPI: Prepulse inhibition; TSH: Thyroid-stimulating hormone; VABS: Vineland Adaptive Behavior Scales; VAS: Visual analog scale.

4.1 Fenobam

Like MPEP, fenobam (*N*-(3-chlorophenyl)-*N*′-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl)urea) is a selective and potent non-competitive mGluR5 antagonist [83] that was studied as a novel, non-benzodiazepine anxiolytic in the 1980s [84]. While fenobam demonstrated efficacy and safety for anxiety, similar to diazepam, in a 5-week, double-blind, placebo-controlled study of 29 outpatients [84], another Phase II trial reported no anxiolytic effect and psychostimulant-like side effects, including hallucinations, vertigo, paresthesias and insomnia, and further development of fenobam as an anxiolytic was discontinued [83]. Porter more recently found that fenobam and MPEP bind the same allosteric modulatory site of the mGluR5 receptor and have similar mechanisms of action [83]. With emergent interest in the role of mGluR5 activity in a number of neuropsychiatric disorders, fenobam is again being investigated as a potential therapeutic agent for anxiety [83], as well as FXS [81,85]. In rodent models of anxiety, fenobam had a potent anxiolytic effect [83]. *Fmr1* KO mice treated with fenobam also demonstrate rescue of the immature dendritic spine morphology in hippocampal neurons [81], as well as impaired procedural memory formation and avoidance behavior [86]. Of note, however, wild-type (WT) mice treated with fenobam showed severe impairment in motor learning, highlighting the importance of accurate diagnosis in treating intellectually impaired individuals with mGluR5 antagonists [86].

A pilot open-label, single dose trial of fenobam in adults with FXS was conducted to evaluate safety and pharmacokinetic properties in human subjects [85]. Outcome measures included performance on a prepulse inhibition (PPI) task and the Carolina Fragile X Project Continuous Performance Test (FXCPT) commission scores, chosen to assess the phenotypic features of sensory gating, attention and response inhibition. Twelve verbal subjects (six men and six women) were administered a single oral dose of fenobam – 50, 100 or 150 mg – with the first subject of each gender receiving 50 mg, the second 100 mg and all subsequent subjects 150 mg. Dosing was based on previous trials for anxiety, with 150 mg representing the middle dose range in which central nervous system (CNS) side effects were observed. Subjects completed the PPI and FXCPT tasks 60 min later; side effects and fenobam plasma concentrations were assessed over the course of 6 h. Fenobam concentration in plasma peaked at 2 – 3 h after dosing, though changes in PPI were determined earlier at 1 h post-dose, possibly underestimating any therapeutic effect. The pharmacokinetic properties of fenobam show wide intersubject variability and suggest that effective dosing may be highly variable as well. Fifty percent of subjects (four men and two women) met the predetermined response criterion for improvement in sensory processing, defined as 20% improvement from baseline in the PPI, a proportion significantly greater than would be expected by chance alone. No significant effect for attention and inhibition was observed on the FXCPT due to low rates of commission errors at baseline and ceiling effects. A single dose of fenobam was well tolerated, with three subjects experiencing mild sedation, and nine subjects showing 'calmed' behavior with improvement in symptoms of impaired eye contact, impaired social interaction, anxiety and hyperactivity. This observed clinical improvement indicates that different behavioral outcome measures are needed to fully characterize the effect of treatment. Conclusions could not be drawn about CNS side effects that emerged in previous studies with chronic dosing in adult subjects with anxiety disorders. As in other open-label studies, results should be interpreted cautiously, as a large placebo effect and observer bias may overestimate the apparent efficacy of fenobam. Randomized, double-blind, placebo-controlled trials are the gold standard in clinical trial design and minimize the influence of such biases, and rigorous controlled trials would be required to draw more definitive conclusions regarding therapeutic effect.

4.2 AFQ056

AFQ056 is a selective, non-competitive mGluR5 antagonist with a short half-life that is currently in clinical trials for the treatment of L-DOPA-induced dyskinesia in Parkinson's disease, Huntington's disease and FXS [82]. AFQ056 has been shown to rescue deficits in PPI and the elongated dendritic spine morphology in cultured hippocampal neurons from *Fmr1* KO mice without causing adverse effects in WT mice [82]. Jacquemont *et al.* recently reported on a Phase II randomized, double-blind, crossover study of AFQ056 in 30 male subjects with FXS aged 18 – 35 years with the Aberrant Behavior Checklist-Community Edition (ABC-C) sum score as a primary outcome measure [87]. As a group, subjects did not show overall improvement on the ABC-C or a number of secondary outcome measures after 19 – 20 days of treatment, with the exception of a modest reduction in repetitive behavior. However, a subgroup of subjects (n = 7) with full methylation of the *FMR1* promoter and no detectable *FMR1* mRNA improved significantly on the ABC-C ($p < 0.001$), Including subscales for Stereotypy, Hyperactivity and Inappropriate Speech, while those with partial promoter methylation (n = 18) showed no response. The subpopulation with full methylation also showed improvement on secondary measures of clinician-rated improvement, including stereotypic behavior, restricted interests and autistic behaviors, but not overall adaptive functioning. Twenty-four of 30 subjects (80%) reported mild to moderate adverse events, most commonly fatigue and headache. Results from a follow-up study suggest that full methylation status may predict AFQ056 response, though as expected, AFQ056 does not have any effect on degree of *FMR1* methylation or transcription *in vitro* [88].

Phase II randomized, double-blind, placebo-controlled trials of AFQ056 in both adolescents and adults with FXS are underway to assess safety, tolerability and efficacy; 2-year open-label extension studies are also planned [89-92]. Novartis is also sponsoring a Phase I open-label study of

pharmacokinetics, safety and tolerability of single and multiple oral doses of AFQ056 in children aged 3 – 11 years with FXS [93].

4.3 Acamprosate

Acamprosate (calcium acetylhomotaurine) is a drug with putative mGluR5 antagonism approved by the FDA for the maintenance of abstinence from alcohol in individuals with alcohol dependence [94]. Characterization in animal models indicates that acamprosate may also act as a weak NMDA receptor antagonist and $GABA_AR$ agonist and have anti-oxidant effects [94]. In an open-label, pilot study of three males aged 18 – 23 years with full mutation FXS and autistic disorder, all subjects demonstrated significant improvement in expressive and pragmatic use of language, though change in non-verbal communication, including eye gaze, was not observed [94]. Two of the three subjects developed dose-dependent nausea and/or emesis, and one subject reported sedation. Observations suggest a positive therapeutic effect, though the small sample size and open-label nature of the study limit any definitive conclusions. While acamprosate is widely available with documented safety in humans, disadvantages include poor oral bioavailability, the need for high and frequent dosing and significantly less potency at mGluR5 in comparison with the prototypical mGluR5 antagonist MPEP [94]. Activity at NMDA and $GABA_ARs$ rather than at mGluR5 receptors may be responsible for its observed therapeutic effect [94]. A Phase III open-label trial of acamprosate (NCT01300923) for the treatment of behavioral, cognitive and social deficits in children aged 5 – 17 with FXS is currently underway at Indiana University [95].

4.4 mGluR5 antagonists in development

RO4917523 (Hoffman-LaRoche, South San Francisco, CA, USA) is an mGluR5 antagonist that has recently been investigated for efficacy in treatment-resistant depression and as an adjunctive treatment for adults with major depressive disorder (MDD) and incomplete response to an antidepressant (NCT00809562; NCT01437657) [96]. Phase II trials in male adults with FXS are currently underway to assess safety, tolerability, pharmacokinetics and efficacy with daily dosing over 6 – 12 weeks (NCT01015430; NCT01517698) [96]. Another mGluR5 antagonist, STX107 (Seaside Therapeutics, Cambridge, MA, USA), has completed Phase I single-dose trials in healthy male adult volunteers (NCT00965432), and Phase II trials are planned to assess tolerability in adult males with FXS (NCT01325740) [97]. No data are publicly available at the time of this report.

2-Chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1*H*-imidazol-4-yl)ethynyl)pyridine (CTEP) is a recently developed, highly selective mGluR5 antagonist with potency 30- to 100-fold greater than MPEP and fenobam *in vivo* [98]. CTEP confers the advantages of bioavailability approaching 100%, as well as a long half-life of 18 h in mice, enabling sustained receptor blockade with dosing once every 48 h [98]. In young adult *Fmr1* KO mice, CTEP has been shown to rescue biochemical, structural and behavioral aspects of the mutant phenotype, including learning and memory impairment, hypersensitivity to an auditory stimulus, susceptibility to seizure, motor hyperactivity, macroorchidism (partial rescue), abnormal dendritic spine density in the visual cortex, increased hippocampal LTD, increased synaptic protein synthesis and increased signaling through ERK and mTOR signaling pathways [99]. Amelioration of multiple fragile X features in young adulthood, after the phenotype is fully established, is particularly notable and implies that correction of an imbalance in synaptic signaling can substantially improve outcome [99]. No trials of CTEP in human subjects have been reported.

5. Other glutamatergic agents

5.1 Riluzole

Riluzole is a drug with unknown exact mechanism of action that is believed to modulate glutamatergic and GABAergic neurotransmission via inhibition of presynaptic glutamate release, enhanced glutamate reuptake, potentiation of postsynaptic $GABA_AR$ activity and blockage of GABA reuptake [100]. Riluzole is FDA-approved for the treatment of amyotrophic lateral sclerosis (ALS), and preliminary studies suggest efficacy for treatment-resistant depression and obsessive–compulsive disorder (OCD) [100]. Riluzole was studied in six young adult males (aged 19 – 24 years) with FXS, autism and moderate ID, with primary outcome measures including the ABC subscales and Children's Yale-Brown Obsessive Compulsive Scale modified for Pervasive Developmental Disorders (CY-BOCS-PDD), the latter intended to capture any change in repetitive, compulsive behaviors [100]. One of six subjects (16%) responded to treatment with a reduction in repetitive behaviors and irritability; there was no significant change on any secondary measures. Despite a lack of clinical response, ERK activation, known to be delayed in FXS and considered a potential biomarker of treatment response, decreased significantly in all patients. Liver function tests increased asymptomatically in five of six (83%) subjects.

5.2 Memantine

Memantine is a non-competitive NMDA receptor antagonist with weak receptor affinity that causes blockade at low synaptic glutamate levels and release of blockade at high glutamate levels and may have a neuroprotective effect [101]. Overactivity of the excitatory glutamate system and underactivity of inhibitory GABAergic pathways have been proposed as causative factors in autism, and memantine may function to ameliorate this imbalance [102]. Approved for the treatment of moderate to severe Alzheimer's dementia and off-label use of memantine for social deficits, cognitive and behavioral symptoms in ASD have been reported, with greatest improvement seen in the areas of language and social behavior [101]. Memantine has been shown to promote maturation of dendritic spines and stimulate excitatory synapse formation in cultured cerebellar granule cells from *Fmr1* KO mice [102].

Erickson *et al.* conducted a chart review of six young adult patients (aged 13 – 22 years) with FXS and a comorbid diagnosis of ASD treated with memantine for a mean duration of 34.7 weeks. Four of six subjects (67%) showed significant clinical improvement as measured by the Clinical Global Impressions-Improvement (CGI-I) score, though specific symptom rating scale scores did not change [101]. Two of six subjects (33%) developed treatment-limiting irritability. Of those tolerating memantine, the greatest gains were reported in social behavior, inattention and irritability. In a separate case report, one 65-year-old female premutation carrier with FXTAS demonstrated a 50% reduction in FXTAS symptoms, including head and hand tremor, ataxia, neuropathy, depressed mood and anxiety with the addition of memantine to venlafaxine [103].

6. Drugs affecting intracellular signaling pathways and target proteins

6.1 Minocycline

Minocycline, a tetracycline derivative antibiotic that inhibits matrix metalloproteinase-9 (MMP-9) and may reduce CNS inflammation, has been studied as a treatment for stroke, multiple sclerosis (MS), amyotrophic lateralizing sclerosis (ALS), Huntington's disease, Parkinson's disease, Alzheimer's disease and autism [104,105]. MMP-9 is involved in normal synaptic structure and function via its role in remodeling the extracellular matrix and is overexpressed in response to mGluR activation [106,107]. Elevated levels of MMP-9 are found in *Fmr1* KO hippocampal neurons and have been linked to immature dendritic spine morphology [104]. Treatment of *Fmr1* KO mice with minocycline restores mature dendritic spine formation, leads to reduced anxiety and more strategic exploratory behavior in maze tasks, and increases mating-related ultrasonic vocalizations, a measure of social communication, to levels comparable with WT controls [104,108]. Minocycline has also been found to restore normal synaptic structure in *Drosophila dfmr1* mutants [107].

Paribello *et al.* conducted a Phase II open-label study of minocycline in 20 adolescent and young adult subjects with FXS treated with 100 mg or 200 mg daily for 8 weeks [106]. Minocycline significantly improved ABC-C Irritability subscale scores, CGI-I scores and the visual Analog Scale for Behavior (VAS). Improvements were also seen in ABC subscales for Stereotypy, Hyperactivity and Inappropriate Speech. Two subjects asymptomatically seroconverted to a positive ANA. Other side effects were mild and included dizziness, diarrhea, drowsiness and headache. Children were not included in the study, as minocycline can cause permanent tooth discoloration during tooth development in childhood. As with other open-label studies, results must be interpreted cautiously. A randomized, placebo-controlled trial of minocycline in children under age 13 with FXS was recently completed [109], though results are not yet reported. A survey of side effects in 50 individuals with FXS treated with minocycline revealed that gastrointestinal (GI) symptoms were most common, including loss of appetite, GI upset and diarrhea [110].

6.2 Lithium

Lithium, traditionally used as a mood-stabilizing agent in bipolar disorder, has several known mechanisms of action, including inhibition of the metabolic regulatory enzyme glycogen synthase kinase-3 (GSK3), which is overactive in the absence of FMRP [111-113]. Treatment with MPEP in *Fmr1* KO mice also results in reduced GSK3 activity, suggesting that mGluR5 antagonists and direct GSK3 inhibitors affect a common signaling pathway and lending support to GSK3 as a potential therapeutic target [112,114]. GSK3 regulates a variety of neuronal substrates, including transcription factors, cell signaling cascades and structural proteins involved in cell architecture, such as (MAP1B) microtubule-associated protein 1B [113]. GSK3 promotes activation of MAP1B, and lithium's inhibitory effects on this system could theoretically correct aspects of the fragile X phenotype linked to upregulated MAP1B activity in *Drosophila* [112]. Lithium has also been shown to increase brain-derived neurotrophic factor (BDNF), a regulator of learning, memory and synaptic plasticity, in *Fmr1* KO mice [114]. *Fmr1* KO mice treated with lithium show amelioration of increased audiogenic seizures, hyperactivity, social preference differences, anxiety-related behaviors in social interactions, learning and memory deficits, abnormal dendritic spine length and density, mGluR-mediated LTD in hippocampal neurons and increased cerebral protein synthesis in the limbic system and hypothalamus [112-117]. Restoration of normal hippocampal LTD persisted in *FMR1* KO mice with long-term treatment of lithium into adulthood, suggesting benefits for treatment throughout the lifespan [116].

Following evidence of improvement in cognitive and behavioral abnormalities in fragile X animal models treated with lithium [75,112,114], Berry-Kravis *et al.* conducted an open-label pilot study of lithium in 15 subjects with FXS, aged 6 – 23 years [111]. Though improvement in ABC-C Irritability subscale scores, the primary outcome measure, was not significant, subjects were rated as significantly improved in ABC-Hyperactivity and Inappropriate Speech subscale scores, CGI, VAS and Vineland Adaptive Behavior Scales (VABS)-Maladaptive Behavior subscale scores. Overall improvement fell in the mild to moderate range. Eleven of 15 subjects reported polyuria and/or polydipsia, and 4 subjects developed elevated thyroid-stimulating hormone (TSH). ERK activity, a potential biomarker for changes in cell signaling, normalized for the 11 subjects tested following treatment with lithium. The authors noted that lithium's diverse biochemical activities and general mood-stabilizing properties may account for some of the observed improvements. Lack of a placebo comparison group and double blinding in this open-label study increases the likelihood that results were influenced by observation and placebo bias and should be interpreted cautiously.

7. GABA receptor agonists

7.1 Arbaclofen

Arbaclofen (STX209; Seaside Therapeutics), the *R*-enantiomer of baclofen, is a $GABA_BR$ agonist that inhibits presynaptic release of glutamate, thereby indirectly reducing downstream signaling of mGluR5 [118]. The therapeutic effect of GABAergic agents may also extend from reduction of the imbalance between excitatory and inhibitory neurotransmission in FXS. *Fmr1* mutant mice show reduced audiogenic seizures during acute treatment with arbaclofen, though tolerance to anticonvulsant effects develops rapidly with treatment extended beyond a single dose [119-121]. In *Fmr1* KO mice, arbaclofen has also been shown to reduce excessive protein synthesis, decrease dendritic spine density to WT values and correct aberrant AMPAR endocytosis [121]. The restoration of normal spine density occurred after weaning and the peak period of new spine development, suggesting that arbaclofen can correct an established phenotype in FXS mouse models. Berry-Kravis *et al.* recently reported results of arbaclofen in a Phase II randomized, double-blind, placebo-controlled crossover study of 63 patients, aged 6 – 40 years, with FXS [122]. Overall, subjects did not show statistically significant improvement on the primary outcome measure, the ABC-C Irritability subscale. Global improvement as measured by the CGI-I showed a trend toward improvement but also did not reach significance. With *post hoc* subset analysis of the 27 subjects presenting with the most severe ratings of social withdrawal, arbaclofen was associated with significant improvement on global measures, as well as the Vineland II-Socialization raw score and the ABC-Social Avoidance subscale, newly developed for use in FXS. In further *post hoc* analysis with the ABC-Social Avoidance subscale, significant improvements in social interaction were observed for the broader study population. Arbaclofen was well tolerated with no serious adverse events; headaches and sedation were most commonly reported. Phase III randomized, placebo-controlled trials of arbaclofen in children, adolescents and adults with FXS across 20 sites are currently underway; long-term, open-label extension trials are also planned [123,124]. Seaside Therapeutics has also reported positive results from an open-label Phase IIa study of arbaclofen in 32 children with ASD, including significant global improvement on CGI-I and significant improvement on the ABC-C Irritability and Social Withdrawal subscales [123].

8. Ampakines

8.1 CX516

Excessive internalization of AMPA receptors from the postsynaptic membrane in the absence of *FMR1* has been associated with exaggerated LTD, reduced LTP and resultant deficits in learning and memory in FXS [63,71]. Positive modulators of AMPA receptors ('ampakines') prolong depolarization and excitatory postsynaptic potentials (EPSPs), primarily by delaying deactivation of the AMPA receptor [125-127]. In turn, enhanced depolarization of the postsynaptic membrane unblocks voltage-sensitive NMDA receptors, which may ultimately account for enhanced induction of LTP [127]. As LTP is thought to be involved in memory encoding, drugs that positively modulate AMPAR activity and thus indirectly facilitate LTP could potentially strengthen some types of memory and improve memory impairment [126,128]. Ampakines have also been shown to increase production of BDNF, which may offer protection from excitotoxic neuronal damage and promote synaptic maturation [127].

CX516 (1-(quinoxalin-6-ylcarbonyl)piperidine; Cortex Pharmaceuticals Irvine, CA, USA) and related ampakines have been shown to improve memory encoding in rats in a number of behavioral paradigms in association with increased hippocampal neuronal firing [128-130]. CX516 also enhanced delayed recall of nonsense syllables in elderly subjects [131] and improved performance on measures of attention and memory in adults with schizophrenia concomitantly treated with stable doses of clozapine [132]. Based on these promising results, CX516 was investigated in a Phase II, randomized, placebo-controlled trial in 49 adults with FXS and ID [133]. While CX516 was well tolerated with no serious adverse events other than a substantial rash in one patient, those taking CX516 did not demonstrate any improvement compared with placebo on a variety of cognitive and behavioral measures during the 4-week investigation period. However, the relatively brief period of monitoring may have precluded detection of more gradually developing cognitive benefits. In a study of elderly subjects, blood concentrations of CX516 were noted to drop by more than 50% between tests administered at 75 and 135 min after drug ingestion [131]. Furthermore, CX516 is a relatively weak ampakine, with primate studies suggesting that a dosage three to four times that used may have been needed to observe a significant effect [133]. The study was somewhat limited by choice of cognitive measures, some of which proved too difficult or complex to provide meaningful results for this population with an average Full Scale Intelligence Quotient (FSIQ) of 43.

9. Oxytocin

Oxytocin (OT) is a neuropeptide synthesized in the hypothalamus that promotes uterine contractions during parturition and milk let-down, as well as prosocial behaviors, including bonding, attachment and sexual behavior. OT also has anxiolytic and stress-reduction effects, and receptors for OT are found throughout the limbic system. Deficits in social interaction associated with autism may be related to aberrant functioning in the OT and related vasopressin systems, and several studies have reported that administration of synthetic OT can improve social behavior [134-136] and repetitive behaviors [137] in individuals with ASD.

Hall *et al.* recently reported results of a small, randomized, double-blind, placebo-controlled, single-dose trial of intranasal OT for the treatment of social anxiety in 10 young adult males with FXS [138]. A small but significant increase in eye

gaze frequency during a structured social challenge task was observed with a dose of 24 IU, and salivary cortisol levels decreased at 48 IU. OT was well tolerated with no adverse effects or changes in heart rate. The authors propose that OT may be effective in reducing social anxiety in FXS, potentially by reducing amygdala reactivity to perceived social threat, decreasing hypothalamic–pituitary–adrenal (HPA) axis activation, and promoting social motivation.

10. Acetylcholinesterase inhibitors

10.1 Donepezil

Aberrant activity in cholinergic pathways may contribute to cognitive impairments frequently observed in FXS, including executive dysfunction and deficits in learning and memory [139]. Kesler *et al.* identified a significantly lower right choline/creatine ratio in the dorsolateral prefrontal cortex (DLPFC) using ^{1}H magnetic resonance spectroscopy in nine males with FXS and a trend toward significantly reduced absolute choline levels [139]. In a 6-week, open-label trial of the acetylcholinesterase inhibitor donepezil, subjects showed improvement in working memory, mental flexibility, hyperactivity, irritability and inattention [139]. A randomized controlled trial of donepezil in young adults with FXS is currently underway at Stanford University (NCT01120626) [140].

11. Conclusions

FXS is the most common single-gene cause of ID and autism with characteristic cognitive, physical and behavioral manifestations. Severe reduction or absence of FMRP results in loss of translational inhibition and overexpression of proteins involved in synaptic plasticity, purported to account for these pathognomonic signs and symptoms. Targeted drug treatments in animal models and humans antagonize group 1 mGluR activity, downstream signaling cascades or upregulated target proteins in an attempt to restore normal synaptic plasticity. Related targets for intervention include presynaptic GABAR and postsynaptic NMDAR and AMPAR activity. Large, well-designed placebo-controlled clinical trials in FXS are lacking and are needed to clarify early findings. The results have important implications for a number of neuropsychiatric disorders that may share clinical features and affected signaling pathways, including autism and a subset of ID.

12. Expert opinion

FXS is a single-gene disorder with neurobiological deficits that are increasingly well understood. In the absence of FMRP acting as a translational brake at excitatory synapses, group 1 mGluRs are overactive, resulting in excessive protein synthesis, enhanced LTD and loss of AMPARs and NMDARs from the synaptic membrane. Like schizophrenia and autism, FXS has been characterized as a 'synapsopathy', a developmental disorder of synaptic development and plasticity [79], with associated imbalance between the excitatory glutamatergic and inhibitory GABAergic systems. The proposed mechanism of disease in FXS affords an exciting opportunity for a targeted treatment approach to a neurodevelopmental disorder with psychiatric manifestations, as the pharmacological treatment of mental illness has traditionally relied on a 'top down', serendipitous approach to drug development, resulting in symptom relief but less often correction of the underlying deficit. Targeted therapeutics in FXS thus have important implications for many other disorders with overlapping symptom clusters, including autism, ID, ADHD and anxiety disorders, as some of these disorders may share affected pathways. Autistic symptoms are particularly common in FXS, and many of the drugs in FXS Phase II and III trials, including OT, memantine and arbaclofen, show promise for the treatment of core symptoms of ASD, as well. While multiple genetic variations have been linked to the development of autism, a subset of individuals with ASD may have a disturbance of glutamatergic transmission similar to fragile X that is also amenable to therapeutics targeting the mGluR5 pathway.

In animal models of FXS, data supporting the mGluR theory and drugs that correct mGluR overexpression are robust, with many studies reporting phenotypic 'rescue' and behavior that is indiscernible from WT. In human trials, results of targeted treatment trials have been encouraging but somewhat less striking. Some novel therapeutics, such as the ampakine CX516, appeared promising in preclinical trials but failed to demonstrate efficacy in clinical trials. The differences observed between animal and human subjects may reflect the milder fragile X phenotype observed in *Fmr1* KO mice, which show less impairment in learning and memory than their human counterparts. Models of neuropsychiatric symptoms in animals are also unlikely to fully represent the complexity of human behavior and experience, and differences in pharmacokinetics, metabolism and dosing protocols between animals and humans could impact outcomes. Future development of targeted treatments should also consider potential unwanted effects based on their neurobiological mechanism of action. Drugs inhibiting overexpressed synaptic proteins, for example, could have off-target effects and cellular toxicity due to participation of these proteins in multiple cellular signaling pathways [57].

In preclinical trials, mGluR5 antagonists have raised excitement for their ability to not only improve maladaptive behaviors but to reverse aspects of the physical phenotype, such as dendrite morphology, and to correct these abnormalities into adulthood. Still, aberrant brain development is present from the earliest stages of life, and some aspects of the phenotype are unlikely to improve with late intervention. One can assume that the most favorable outcomes would be obtained with early childhood intervention, and the choice of target population in future clinical trials should be carefully considered [79]. Drugs that alter glutamate transmission and synaptic protein synthesis may improve cognitive functioning

in individuals with FXS but lead to cognitive impairment in healthy volunteers without similar deficits, and this differential effect should also be considered during analysis of Phase I trials for newly developed drugs [79].

Clinical trials in FXS have been hampered by methodological challenges. The fragile X population is relatively small, and recruitment of subjects to adequately power randomized controlled trials is difficult, even in multi-site studies. The majority of completed trials have used an open-label study design that carries significant potential for bias, particularly placebo and observer bias, and increased risk of type II error. Furthermore, many potential subjects with FXS have significant emotional and behavioral symptoms and are excluded from participation in Phase II and III drug trials because they are already taking psychotropic medications. Individuals with FXS have historically been excluded from larger studies of autism, and outcome measures that have been developed for more common neuropsychiatric disorders may lose validity when applied to the specific fragile X phenotype. FXS is uniquely associated with a diverse assortment of physical, psychological and cognitive symptoms, and because it has been unclear which features novel drugs may affect, choosing appropriate outcome measures has been challenging. While some studies have primarily focused on cognitive changes, others have chosen to track specific behavioral measures, such as social relatedness or irritability. Others have included biochemical markers of altered synaptic activity, though change in these markers has not always correlated with significant clinical effect. Failure to capture change in a particular area of functioning due to inadequate outcome measures could represent a missed therapeutic opportunity.

The ABC-C is a behavioral outcome measure with five subscales that has been validated and extensively utilized in ID and autism populations, and a recent exploratory and confirmatory factor analysis examined its psychometric properties in 630 children and young adults with FXS [141]. The findings support modification of the original five factors (Irritability; Lethargy/Withdrawal; Stereotypy; Hyperactivity; Inappropriate Speech), as well as the addition of a new sixth factor, Social Avoidance, to improve sensitivity and specificity in the fragile X population. Further studies are needed to develop new behavioral outcome measures that specifically reflect the FXS phenotype, as well as cognitive measures that are appropriately matched to ability level and can accurately reflect change with intervention.

A number of multi-site randomized, controlled trials to further explore the efficacy of novel medications in FXS are underway, including minocycline, arbaclofen, donepezil and the mGluR5 antagonists AQ056, R04917523 and STX107. A major consequence of group 1 mGluR overactivity is loss of ionotropic NMDARs and AMPARs from the postsynaptic membrane, and future targeted treatment trials should also include drugs with the potential to modulate glutamatergic neurotransmission at these sites. Both reduced and enhanced NMDAR functioning has been associated with ASD-like phenotypes in mice, suggesting that maintaining balanced levels of NDMAR activity is critical [142]. NMDAR partial agonists, including D-cycloserine and the related compound D-serine, bind to the regulatory glycine binding site on NMDARs and enhance activation in the presence of low concentrations of glycine site agonists but inhibit receptor/ion channel complex activity when agonist concentration is high [143]. In a pilot study of D-cycloserine in 10 subjects with autistic disorder, 40% showed meaningful overall improvement, particularly in symptoms of social withdrawal [144]. Mice with ASD-like behaviors and mutation in the *Shank2* gene show markedly decreased NMDAR function and impaired social behaviors, both of which significantly improve with D-cycloserine [142]. Human trials of D-cycloserine in other putative hypoglutamatergic neuropsychiatric disorders, such as schizophrenia, have been less promising, possibly due to D-cycloserine's relatively low affinity for the glycine site [145]. D-Serine, however, is a full agonist at the glycine binding site and may have a more potent therapeutic effect; trials of D-serine in the treatment of prodromal schizophrenia are currently underway (NCT00826202) [146]. GLYX-13 is a more recently developed NMDAR glycine site partial agonist that both enhances LTP and suppresses LTD in the hippocampus *in vitro*, suggesting promise for impairments in learning and memory in FXS and other neuropsychiatric disorders [147]. Compared with D-cycloserine, GLYX-13 is a more potent glycine site agonist and has greater selectivity for NR2B-subunit-containing NMDARs, which may explain its more selective enhancement of LTP [147]. GLYX-13 has been shown to improve pro-social vocalizations in mouse models of autism [143]. Phase I clinical trials have been completed, and a Phase II trial of GLYX-13 as an add-on treatment in MDD is planned [148]. In the design of future *in vivo* studies of GLYX-13, consideration should be given to its differential effects depending on drug concentration. At lower concentrations, it appears to enhance LTP and block LTD, though at higher concentrations, it can impair LTP and lose its effect on LTD [147].

An alternative mechanism for reducing activation of postsynaptic mGluR5s, other than direct antagonism, is stimulation of mGluR2s and mGluR3s, presynaptic autoreceptors that modulate release of glutamate into the synaptic cleft. LY2140023 (pomaglumetad methionil) is a selective mGluR2/3 receptor agonist that demonstrated initial efficacy for reducing psychotic symptoms in schizophrenia similar to currently available antipsychotic drugs, though a larger Phase II clinical trial demonstrated a large placebo response and failed to replicate previous findings [149,150]. LY2140023 appeared well tolerated with the notable exception of increased seizure activity. However, in a subsequent 24-week, open-label, active-controlled study comparing LY2140023 and standard of care treatment with atypical antipsychotics, no seizures in the LY2140023 group were reported [149]. Risk of seizure activity should be further

characterized before considering trials in individuals with FXS, many of whom have a concomitant seizure disorder.

Characterization of the neurobiological defects and targeted drug development in FXS affords an exciting opportunity for the application of translational medicine in the treatment of neuropsychiatric disorders. FXS is the most common known genetic cause of autism and ID, and studies in FXS therefore have potentially important implications for the broader understanding and treatment of these disorders. Future clinical trials are needed to clarify the efficacy of existing targeted drugs for different symptom domains, explore the utility of recently developed drugs that impact glutamatergic neurotransmission in novel ways and define outcome measures that promote consistency and allow comparison across trials.

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Declaration of interest

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