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Potential therapeutic interventions for fragile X syndrome

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Abstract

Fragile X syndrome (FXS) is caused by a lack of the fragile X mental retardation protein (FMRP); FMRP deficiency in neurons of patients with FXS causes intellectual disability (IQ<70) and several behavioural problems, including hyperactivity and autistic-like features. In the brain, no gross morphological malformations have been found, although subtle spine abnormalities have been reported. FXS has been linked to altered group I metabotropic glutamate receptor (mGluR)-dependent and independent forms of synaptic plasticity. Here, we discuss potential targeted therapeutic strategies developed to specifically correct disturbances in the excitatory mGluR and the inhibitory gamma-aminobutyric (GABA) receptor pathways that have been tested in animal models and/or in clinical trials with patients with FXS.

Keywords

fragile X syndrome; therapy; metabotropic glutamate receptors (mGluRs); gamma-aminobutyric (GABA) receptor

Fragile X syndrome

Intellectual disability, affecting 1–3% of the population, is represented by an IQ less than 70 and can be caused by nongenetic and genetic factors. Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability affecting approximately one in 4000 males and one in 8000 females¹. In 1991, an expanded trinucleotide repeat in the *FMR1* gene, located on the long (q) arm of the X chromosome at position 27.3, was identified as the genetic cause of FXS². The gene contains a CGG repeat in its 5' untranslated region (UTR) that is normally shorter than 55 repeats³. However, this repeat can become unstable upon maternal transmission, usually resulting in the expansion of the repeat in the next generation. Individuals with repeat sizes between 55 and 200 units long are considered premutation carriers⁴. If the CGG is abnormally repeated from 200 to more than 1000 times, referred to as a full mutation, the CGG repeat and the neighbouring CpG island in the *FMR1* promoter region are hypermethylated⁵. Typically, hypermethylation results in silencing of the *FMR1* gene, leading to FMRP deficiency and intellectual disability in patients with FXS. The vast majority of males carrying a full mutation will develop FXS with cognitive impairments, whereas only 25% of female full mutation carriers present an IQ below 70 owing to the presence of a normal X chromosome that produces FMRP. However, the majority of female patients with FXS do have

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learning and/or social-emotional problems⁶. A few rare affected individuals have been identified that have either a point mutation or a deletion in the *FMR1* gene.

The clinical presentation of FXS varies considerably. In addition to intellectual disability, patients with FXS usually exhibit neurodevelopmental problems including attention deficit hyperactivity disorder (ADHD) and disruptive and autistic-like behaviour^{7–8}. Additional features of FXS include mild facial abnormalities, macroorchidism (see Glossary), sleep problems and epileptic seizures^{9–12}.

FMRP and synapse morphology

FMRP, an RNA binding protein, is ubiquitously expressed but predominates in the neurons of the brain¹³. FMRP binds RNA through its three RNA binding domains (*i.e.* two K homology (KH) domains and one RGG box). FMRP binds to guanine quartet RNA structures and a sequence-specific element that serves as the RNA target for the KH2 domain, forming together the FMRP kissing complex^{3, 14}. FMRP recognition motifs are typically found in the 3' UTR of target mRNAs³. In neurons, FMRP binds to target mRNAs, most likely in the nucleus, including the *FMR1* mRNA^{15–16}. Subsequently, FMRP is thought to repress the translation of these target mRNAs during transport to postsynaptic dendritic spines, where its activity is important for synaptic plasticity (Box 1).

Box 1

FMRP and synaptic plasticity

Consolidation of memory and learning processes are highly conserved throughout evolution. Learning is the process by which we acquire knowledge, whereas memory is the process by which that knowledge is encoded, stored and later retrieved. The study of learning is central to understanding disorders involving intellectual disability, including FXS. Neuroscience has expanded our knowledge about the cellular and molecular mechanisms underlying these processes³⁰. Learning and memory involves the change in the transmission efficacy at the synapse (synaptic plasticity).

Synaptic plasticity is the ability of the connection or synapse between two functional neurons to change in strength. Changes in synaptic strength can be short term (seconds to minutes) or long term (hours and days). Only long-term changes alter the structure of the synapse and these changes are mediated by rapid local protein synthesis in dendrites, particularly in the postsynaptic compartment. FMRP plays an important role in translational control after group I mGluR stimulation. The loss of the FMRP, the defect responsible for FXS, has been linked to several functional deficits in synaptic plasticity, including weakening of synaptic strength. A model was proposed in which FMRP represses the translation of specific mRNAs at the synapse³². FMRP thus acts as the “brake” on the protein-synthesis dependent functions of mGluR5 activation, including strengthening of the synapse. In the absence of FMRP, mGluR5 activation is exaggerated and synaptic strength is weakened and this is the proposed basis of the intellectual disability in patients with FXS.

Spines are specialized dendritic protrusions and rapid changing; creation and elimination of these protrusions are essential for learning and memory¹⁷. Compelling evidence shows that local mRNA translation at synapses plays an important role in neuronal processes (Box 2) including the modulation of synaptic plasticity¹⁸. The presence of the protein producing machinery near synaptic connections allows neurons to rapidly respond to synaptic activity through the local translation of specific mRNAs¹⁸. As a consequence of local mRNA translation and synthesis of specific proteins, including FMRP, the protein content of the

postsynaptic compartment can be remodelled locally, thereby changing the functionality and morphological structure of the synapse.

Box 2

mRNA transport and local translation

Local mRNA translation in the spines plays an important role in neuronal processes, including the modulation of synaptic plasticity¹⁸. The presence of the protein machinery near synaptic connections allows neurons to rapidly respond to synaptic activity through local translation of specific mRNAs. Therefore, efficient transport of specific mRNAs in a translationally silent state to synapses is followed by controlled efficient translation at the postsynaptic compartment upon stimulation. The repository of locally translated mRNAs is broad and includes: Map1b, CaMKII α , Arc and Fmr1. These dendritic mRNAs have special sequences or motifs usually located at the 3'UTR called dendritic targeting elements (DTEs)¹⁰². RNA-binding proteins recognize this motif to form RNA transporting granules, which are transported along microtubules to reach the synapse¹⁰³. FMRP is such an RNA-binding protein and many mRNAs are targets of FMRP. As a consequence of local mRNA translation and synthesis of specific proteins, remodelling of the protein content of the postsynaptic compartment occurs, thereby changing the functionality and structure of the synapse. Concerning the FXS phenotype, Arc is an interesting target because it has been linked to AMPA receptor internalization^{34–35}. After stimulation of group I mGluR, Arc is locally synthesized and is involved in the rapid internalization of AMPA receptors. In the absence of FMRP, Arc protein expression might be increased, resulting in excessive AMPA receptor internalization and immature spine morphology. Together with Arc, other proteins, such as Map1b are misregulated in FXS and seem to contribute to the FXS phenotype⁸⁸.

Microscopic analysis of brain material from both patients with FXS and *Fmr1* knockout (KO) mice reveals no gross morphological abnormalities^{19–20}. However, in specific brain areas long and thin dendritic spines have been observed, consistent with an immature spine phenotype^{21–24}. The discovery of a morphological spine phenotype indicates a possible defect in synaptic plasticity in FXS that could result in the intellectual disability phenotype. Whether the abnormal spine morphology is a cause or a consequence of altered signal transmission is currently unknown. Interestingly, several other intellectual disability syndromes including Down syndrome and Rett syndrome also show an altered spine phenotype^{25–26}.

The mGluR theory

In the brain, two major classes of neurotransmitter receptors are present at the synaptic membrane and can be divided into ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels, and binding of a specific ligand induces a conformational change that leads to the opening of the receptor pore. The open receptor permits ionic influx across the cell membrane changing the excitability of the neuron. AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartic acid) and kainate receptors form the major class of ionotropic glutamate receptors in the brain, mediating fast excitatory neurotransmission. Metabotropic receptors (mGluRs) have a seven helix-containing transmembrane domain (7TMD). They act by a G protein-dependent mechanism, hence are also referred to as G-protein-coupled receptors (GPCR); however, they can also transduce signals concomitantly through G-protein-independent mechanisms. G proteins extrapolate extracellular signals into an intracellular response. The family of metabotropic glutamate receptors comprises eight different subtypes (mGluR1–8) that are divided into three distinct groups (i.e. group I, II and III) on the basis of sequence similarities and pharmacological properties. Group I includes mGluR1 and mGluR5 receptors, which couple to the Gq protein

and activate phospholipase c (PLC) ²⁷. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, Glu7 and Glu8) receptors couple to Gi/Go protein and inhibit adenylyl cyclase ^{28–29}.

Long-term potentiation (LTP) and long-term depression (LTD) are considered the major cellular mechanisms underlying synaptic plasticity, defined as long-lasting alterations in synaptic strength accompanied by alterations in spine size and morphology ³⁰. LTP is the strengthening of the connection between a presynaptic and postsynaptic neuron. LTD is the antithesis of LTP and is defined as the weakening of the synapse, and is mainly reflected by a reduced number of ionotropic glutamate responsive AMPA receptors at the postsynaptic membrane ³¹. In 2004, Bear and colleagues proposed the mGluR theory to explain many aspects of the clinical symptoms found in patients with FXS and in the *Fmr1* KO mouse including: (i) higher density of spines and more immature spines as compared to normal individuals or wild-type mice; (ii) electrophysiological deficits in *Fmr1* KO mice; (iii) exaggerated dendritic protein synthesis in *Fmr1* KO mice after the activation of mGluR5; and (iv) behavioural phenotypes in patients with FXS and *Fmr1* KO mice ³². This mGluR theory states that AMPA receptor internalization, triggered by group I mGluR stimulation (mGluR1 or mGluR5) is exaggerated in FXS (Figure 1). Stimulation of group I mGluR by glutamate induces local mRNA translation, resulting in novel protein synthesis that subsequently stimulates the internalization of AMPA receptors, which are essential for long-term plasticity. *FMR1* mRNA is also present in the postsynaptic compartment and FMRP is locally synthesized upon mGluR activation ³³. FMRP negatively regulates the translation of proteins that are important for AMPA receptor internalization (Box 2) ^{34–35}. The model predicts that in the absence of FMRP, the increased translation of a subset of mRNAs perturbs receptor internalization dynamics, thereby exaggerating internalization of AMPA receptors and weakening the synapse. Indeed, mGluR LTD is enhanced in the *Fmr1* KO mouse model compared to wild-type control ³⁶. Moreover, in wild-type mice mGluR LTD is protein synthesis dependent, whereas in the *Fmr1* KO mice it is protein synthesis independent. This suggests that in absence of FMRP, proteins that are important for the maintenance of mGluR LTD are already abundantly present at the synapse. Overall, support for the mGluR theory has come from many studies including an elegant study demonstrating that *Fmr1* KO mice with a 50% reduction in mGluR5 expression exhibit specific phenotypic rescues, including the immature spine phenotype, elevated protein synthesis at synapses and fewer audiogenic seizures compared to the *Fmr1* KO mouse ³⁷.

The exact mechanism by which FMRP represses local mRNA translation is still under investigation. FMRP can regulate synaptic translation at the level of initiation through recruitment of CYFIP1/Sra1. Reduction of CYFIP1 in neurons results in increased level of proteins encoded by known FMRP target mRNAs ¹⁸. In addition, phosphorylation and dephosphorylation of FMRP seems to play a crucial role in the downstream signalling cascade induced by mGluR5 stimulation, which ultimately leads to local protein synthesis at the synapse (Figure 2). FMRP can be phosphorylated specifically at serine 499 ³⁸. The phosphorylation status of FMRP influences the translation of target mRNAs because phosphorylated FMRP is associated with stalled polyribosomes (repression of translation) and unphosphorylated FMRP is associated with actively translating polyribosomes ³⁸. Subsequently, upon group I mGluR stimulation, FMRP is rapidly dephosphorylated by protein phosphatase 2a (PP2a), allowing rapid mRNA translation ³⁹. Simultaneously, the activation of the mammalian target of rapamycin (mTOR) cascade results in the phosphorylation of S6 kinase, leading to the rephosphorylation of FMRP ⁴⁰ (Figure 2). The mTOR signalling pathway might play a prominent role in FXS, because the mTOR signalling pathway in the hippocampus is overactivated in *Fmr1* KO mice leading to aberrant synthesis of synaptic proteins and exaggerated protein synthesis-dependent mGluR-LTD ⁴¹.

Since the formulation of the mGluR theory, clinicians in the FXS field have partnered with the pharmaceutical industry to search for therapeutic strategies aimed at mGluR5, which could be a valid target through which a partial reversal of some of the features of FXS in patients can be realized. MPEP (2-methyl-6-(phenylethynyl)-pyridine), a negative mGluR5 modulator, can counteract the excessive activity of the mGluR5 and rescue the net loss of AMPA receptors after loss of FMRP *in vitro*⁴². Pharmacological rescue has been established for audiogenic seizures, behavioural phenotypes and spine abnormalities using several negative mGluR5 modulators in FXS animal models and will be described below in more detail.

The mGluR theory has directed research towards the basic mechanisms underlying FXS and led to the initiation of novel therapeutic strategies.

GABA hypothesis

In addition to the mGluR theory, it is also hypothesized that gamma-aminobutyric (GABA) receptor signalling is altered in patients with FXS⁴³. GABA is the major inhibitory neurotransmitter in the central nervous system (CNS) and as such plays a key role in modulating neuronal activity. GABA mediates its action via two distinct receptor systems, the ionotropic GABA_A and metabotropic GABA_B receptors. Many patients with FXS suffer from epilepsy and sleeping problems, which are linked to GABA receptor signalling⁴³. Interestingly, mRNAs encoding GABA_AR subunits are targets of FMRP⁴⁴. *Fmr1* KO mice express decreased mRNA and protein levels of several GABA_AR subunits (GABA_AR α 5, β 2 and δ) compared to wild-type littermates^{45–48}. These studies support a model in which FMRP controls the stability of GABA_AR subunit mRNAs and prevents their degradation by binding these mRNAs *in vivo*. In addition, reduced mRNA expression of the GABA synthesizing enzyme glutamate decarboxylase (*GAD67*) has been reported in *Fmr1* KO mice compared to wild-type mice, although, another study showed increased *GAD67* protein expression in *Fmr1* KO mice⁴⁹. The altered expression of GABA signalling in *Fmr1* KO mice reflects: (i) decreased GABA receptor signalling efficiency in the hippocampus of the *Fmr1* KO mouse⁵⁰, (ii) downregulation of tonic GABA receptor inhibition⁴⁷ and (iii) morphological defects of GABA releasing interneurons in the neocortex⁵¹ compared to wild-type mice.

In addition to GABA_AR, GABA_BR might also play a role in FXS and, therefore, might be a therapeutic target. GABA_BR agonists inhibit presynaptic glutamate release and inhibit the postsynaptic signalling cascade downstream of mGluR5^{52–53}. Recently, GABA_B deficits were linked to FXS in a study that showed reduced audiogenic seizures in *Fmr1* KO mice after the administration of a GABA_B agonist compared to untreated animals⁵⁴. Also, in light of exaggerated excitatory group I mGluR signalling in FXS, stimulation of the inhibitory pathway might be a good therapeutic strategy to restore the balance between inhibitory and excitatory signalling.

In summary, different studies have reported altered mRNA and protein expression of several GABA_A receptor subunits and points to a GABA receptor dysfunction in the FXS phenotype.

Therapeutic interventions in FXS

To date, treatment of patients with FXS is symptomatic. The two most widely used medications are stimulants that help with attention and hyperactivity and selective serotonin reuptake inhibitors (SSRIs) that can reduce aggression associated with anxiety (www.fragilex.org/html/medications.htm). Patients with FXS are not only treated with pharmacological agents but also seem to benefit from behavioural therapy addressing speech and emotional problems. As demonstrated in the FXS mouse model, an enriched environment can improve behaviour, and thus this therapy might also be beneficial for patients^{55–56}. Current therapeutic strategies, both pharmacological and nonpharmacological, impact symptoms only

and do not improve cognitive function. Recently, new strategies for therapeutic intervention have been developed based on the mGluR and GABA theories (Figure 3). Several clinical trials have been conducted using a variety of existing and new drugs that are designed to correct the abnormal activity of the mGluR or GABA pathways (Table 1).

Negative modulators of mGluR5

MPEP was one of the first negative modulators of mGluR5 identified, but is often referred as an mGluR5 antagonist or inverse agonist. MPEP interacts within the 7TMD region of the mGluR5⁵⁷. MPEP rescues synaptic plasticity, courtship behaviour and mushroom body defects in a *Drosophila* model of FXS⁵⁸. MPEP also has beneficial effects on some specific phenotypes in *Fmr1* KO mice, including suppression of audiogenic seizures and rescue of deficits in prepulse inhibition of acoustic startle (PPI)^{59–60}. The use of MPEP is not feasible in clinical trials for patients with FXS owing to toxicity and a short half life.

During a functional high-throughput screen, fenobam was identified as a highly potent, selective negative modulator of mGluR5⁶¹. In the early 1980 s, fenobam was studied as an anxiolytic drug in several clinical phase II trials. Fenobam was the first negative mGluR5 modulator tested in patients with FXS⁶². Twelve patients (six males and six females) received a single oral dose of fenobam. To test if fenobam had any significant effect on the phenotypes of patients with FXS, PPI was measured before and after the administration of the drug. Patients generally showed a decreased PPI compared to healthy control individuals⁶³. Response criteria for fenobam on PPI levels were based on an improvement of at least 20% over baseline with a 95% confidence interval. With these criteria, six out of twelve patients showed an improvement of PPI after fenobam treatment, ranging from 23.7% to 44.2%. No significant adverse reactions to fenobam were noticed and no safety concerns were found. Although these results look promising, it is difficult to draw final conclusions because the trial was: (i) not placebo-controlled, (ii) patients only received a single dose of fenobam and (iii) the number of participants was too limited.

Novartis, Merck and Hoffmann-LaRoche have designed negative modulators of mGluR5 for treatment of FXS. Novartis recently developed a new specific mGluR5 negative modulator, AFQ056, which is currently in a phase II clinical trial (<http://www.clinicaltrials.gov>). A compound from Merck, called STX107, licensed to Seaside Therapeutics (www.seasidetherapeutics.com), is a small, selective negative mGluR5 modulator and is in phase I clinical trials and in preparation for trials in FXS (<http://www.clinicaltrials.gov>). The compound from Hoffman-LaRoche, called RO4917523, is currently in phase II clinical trial (<http://www.clinicaltrials.gov>). Unfortunately, no data have yet been published about the effects of AFQ056, STX107 or RO4917523 on behaviour or spine deficits in *Fmr1* KO mice.

Animal models have provided knowledge about the effects of negative mGluR5 modulators on behaviour and spine deficits in FXS. MPEP rescued several behavioural deficits in different animal models for FXS. To date several clinical trials with patients with FXS are ongoing using different negative modulators of mGluR5.

GABA_A agonists

Chang and colleagues reported that GABA-related treatment had beneficial effects on *dFmr1* mutant flies⁶⁴. *dFmr1* mutant *Drosophila* died when reared on food that contained increased levels of glutamate. Two compounds, nipocotic acid and creatinine, rescued the glutamate-induced toxicity of *dFmr1* mutant flies. The abnormal mushroom body structure and abnormal courtship behaviour observed in *dFmr1* mutant flies were also restored. Nipocotic acid acts as a GABA reuptake inhibitor and creatinine seems to be a potential activator of the GABA_AR. In addition, both drugs and GABA_AR treatment also rescue (i)

Futsch overexpression, an orthologue of mammalian *Map1b* in *Drosophila*, and (ii) abnormalities in mushroom body structure.

GABA_AR agonists are currently in use as anticonvulsants, antidepressants and anxiolytic compounds. Benzodiazepines, which enhance GABA receptor function⁶⁵, are the best known GABA receptor drugs. Although they have anxiolytic effects and are used as a therapy for patients with FXS, they engender unwanted side effects, such as sedation or ataxia and the cessation of treatment can cause withdrawal symptoms⁶⁶. Currently, more selective GABA_AR agonists that lack unwanted side effects are being investigated⁶⁷. In addition to selective GABA_AR agonists, neuroactive steroids that allosterically modulate GABA_AR might be effective; for instance, ganaxolone has a favourable safety profile and this drug might eventually be evaluated in patients with FXS⁸. However, this class of compounds also has other expected properties of GABA_AR modulators, including anticonvulsant activity and the (unwanted) ability to cause sedation⁶⁸.

Targeting the GABA_AR in patients with FXS will most likely be used to reduce specific symptoms, such as epilepsy or sleep disturbances.

GABA_B agonists

Seizures are one of the most robust and reproducible phenotypes in the *Fmr1* KO mouse and occur in approximately 13%–18% of patients with FXS¹⁰. GPCRs, including mGluR5 and GABA_BR, are implicated as causative factors of audiogenic seizures⁶⁹. Treatment of *Fmr1* KO mice with racemic baclofen reduced the incidence of audiogenic seizures⁵⁴.

Racemic baclofen, composed of arbaclofen and S-baclofen, has been used in clinical practice for spasm, pain and addiction⁷⁰. Arbaclofen (STX209), the R-enantiomer purified from racemic baclofen and a GABA_B agonist, has been studied in a phase II randomized double-blind placebo-controlled crossover trial in patients with FXS (www.seasidetherapeutics.com). Data from this trial are not yet published. Another advantage of GABA_B receptor agonist treatment might be the reduction in anxiety in patients with FXS, because GABA_B receptor plays an important role in anxiety⁷¹. However, similar to most intervention based on the GABA_B receptor, there is a chance that patients will show withdrawal symptoms after discontinuation of baclofen treatment⁷².

Treatment of *Fmr1* KO mice with a GABA_BR agonist can inhibit audiogenic seizures and suggests that GABA_BR is involved in some aspects of the etiology in FXS.

AMPA positive modulator

The increased internalization of AMPA receptors in neurons of *Fmr1* KO mice is thought to play a major role in the altered signal transmission. CX516 is an ampakine (see Glossary) that acts as an AMPA receptor positive allosteric modulator. It binds to the AMPA receptor-channel complex, inducing slower receptor deactivation that results in a longer opening time, slower excitatory postsynaptic potential (EPSP) decay and enhanced hippocampal LTP as compared to the basal status before the drug application⁷³. Consequently, it potentiates AMPA receptors after synaptic activation by glutamate and, therefore, it is expected to increase synaptic strength in the presence of glutamate activation. CX516 was tested in a phase II randomized double-blind, placebo-controlled four-week safety trial in adult patients with FXS⁷⁴. Patients underwent detailed assessment to score cognitive and behavioural outcomes. However, four weeks of treatment with CX516 did not result in significant improvement in either cognitive or behavioural measures. This could result from low doses, the short half-life of CX516 in humans or the short period of treatment. In this study, there were minimal side effects and no serious adverse effects were noted.

Theoretically, targeting the AMPA receptor might improve behaviour in FXS, although beneficial effects of ampakines have not yet been confirmed either in animal studies or in clinical trials.

NMDA receptor antagonists

Memantine is an uncompetitive NMDA receptor antagonist, which can retard the progression of Alzheimer's disease and is tested for treatment of pervasive developmental disorders (PDD, see Glossary)^{75–76}. Binding of memantine to the NMDA receptor blocks receptor signalling at low synaptic glutamate levels; this block is released at high glutamate levels^{77–78}. Pilpel and colleagues found that juvenile *Fmr1* KO mice displayed a significantly lower AMPA to NMDA ratio compared to wild-type mice⁷⁹. The difference in ratio at P14 is caused by an upregulation of the NMDA receptor component and a downregulation of the AMPA receptor component. Furthermore, LTP deficits in specific brain areas of *Fmr1* KO mice are linked to dysregulated NMDA receptor signalling, although conflicting results have been published^{80–83}. In addition to excessive signalling via mGluR5 that is linked to increased AMPA receptor internalization, dysregulation of NMDA receptor activity might also be involved, and therefore memantine might have positive effects on the behavioural phenotypes of patients with FXS.

Erickson and colleagues started a small clinical trial with memantine in six patients with FXS who have a comorbid diagnosis of PDD⁸⁴. The effect of treatment was determined by clinical assessment of a Clinical Global Impressions-Improvement (CGI-I, see Glossary) score during the treatment period. No significant improvement was observed after treatment with memantine, but four out of six patients trended towards improvement. Unfortunately, this study was not a placebo-controlled randomized trial and, thus, it is difficult to draw conclusions from it.

The NMDA receptor might be a pharmacological target for treatment, because some brain regions in *Fmr1* KO mice show impaired NMDA-dependent LTP. However, compelling evidence that NMDA receptor antagonist indeed has beneficial effects on behaviour in FXS mouse model and patients with FXS is lacking.

Additional therapeutic interventions

Lithium has been used for many years as a mood stabilizer⁸⁵. Lithium influences several pathways including: (i) inositol(myo)-1(or 4)-monophosphatase 1 (IMPase) and inositol-depletion pathway; (ii) the glycogen synthase kinase-3 (GSK3) pathway; and (iii) the β -arrestin-2-Akt complex. Most studies that have linked Lithium to FXS have focused on the GSK3 pathway^{86–87}. Lithium inhibits the activity of GSK-3 β , which in turn inhibits the phosphorylation of microtubule-associated-protein 1B (Map1B). *Map1B* is one of the major mRNA targets that is bound by and translationally regulated by FMRP^{87–88}. Different brain areas of *Fmr1* KO mice, including striatum and cortex, show increased GSK-3 activity; Lithium and the mGluR5 modulator MPEP similarly inhibit the activity of GSK-3⁸⁶. In 2008, an open-label treatment trial of Lithium in patients with FXS was published⁸⁹. Although the trial was not a placebo-controlled randomized trial, Lithium seems to have had beneficial effects in patients with FXS (*i.e.* decreased responses of aggression, abnormal vocalizations, self-abuse and anxiety). The outcomes were measured by rating scales and tests, such as the Aberrant Behaviour Checklist-Community (ABC-C, see Glossary) caregiver-rated scale and the CGI-I scale. Significant improvement was found in behaviour (ABC-C and CGI-I) and in cognitive function, assessed by Vineland Adaptive Behaviour Scales (VABS, see Glossary) and Repeatable Battery for the Assessment of Neuropsychological status (RBANS, see Glossary) List Learning. This suggests that the behavioural improvement associates with functional improvements in daily life. Nevertheless, it remains difficult to study improvement in cognitive function in patients with FXS, because exploratory tasks are difficult for lower-functioning

patients. In conclusion, Lithium seems to have beneficial effects on behaviour and in some cognitive functions, but it will be important to investigate more precisely the effects of Lithium by means of a long-term placebo-controlled trial in patients with FXS.

Minocycline is a tetracycline analogue that can inhibit matrix metalloproteinase-9 (MMP-9) and reduce inflammation in the CNS. MMP-9 is an extracellular endopeptidase that cleaves extracellular matrix (ECM) proteins that impact synaptogenesis and spine morphology⁹⁰. Minocycline has been tested in clinical trials for treatments of multiple neurological disorders, including stroke, multiple sclerosis and autism. Recently, minocycline was shown to have beneficial effects on the maturation of spines in cultured hippocampal neurons and in organotypic slices of *Fmr1* KO mice. It can also rescue the anxiety in *Fmr1* KO mice as assessed by an elevated plus maze test⁹¹. Minocycline is believed to impact the mGluR pathway. DHPG (3,5-dihydroxyphenylglycine), a group I mGluR agonist, induces MMP-9 expression and activation upon stimulation of hippocampal neurons⁹¹. Therefore, it is possible that MMP-9 expression and activation is enhanced in neurons from *Fmr1* KO mice owing to increased group I mGluR signalling. This might contribute to abnormal dendritic spine development in *Fmr1* KO hippocampal neurons. Clinical trials have been started for patients with FXS and an open-label trial has been recently completed to study the effects of minocycline in 50 patients with FXS⁹². The caretakers reported an improvement in language and behaviour surveyed by a Likert scale (see Glossary). Currently, children 4–16 years old with FXS are being recruited to be evaluated in a placebo-controlled trial for the efficacy of minocycline (<http://www.clinicaltrials.gov>).

Acamprosate (calcium acetylhomotaurine) is a commercially available drug used for the maintenance of alcohol abstinence⁹³. Acamprosate seems to have many modes of action including as an mGluR5 antagonist, a weak NMDA receptor antagonist and a GABA_AR agonist^{94–95}. Although the exact mechanism of action of acamprosate is not completely understood, Erickson and colleagues conducted a small clinical trial with three patients with FXS⁹⁶. To evaluate response to acamprosate treatment, the CGI-I scale was used. After a minimum of 16 weeks of treatment, all three patients improved. Strikingly, all three patients showed an improvement in language skills. Two subjects experienced nausea with no other adverse effects. Although these results seem promising, this trial was not placebo-controlled and the number of participants was too limited to draw definitive conclusions. In addition, the effect of Aripiprazole on patients with FXS has been tested⁹⁷. Aripiprazole is an atypical antipsychotic that has been approved in the U.S. by the Food and Drug Administration to treat children or adolescents who have autism. The effect of Aripiprazole in patients with FXS was evaluated by CGI-I and ABC-I scale. All subjects who completed the study improved significantly. However, this trial was not placebo-controlled, and thus conclusions are difficult to state.

Other compounds not targeting the mGluRs and GABA receptors might be beneficial for therapeutic use in patients with FXS, although more experiments have to be performed to elucidate whether these compounds are effective. An important first step toward a new therapeutic strategy for FXS that is not based on mGluRs and GABA receptors has been taken. Researchers have found that the activity of phosphoinositide-3 (PI3) kinase was increased in the *Fmr1* KO mice compared to wild-type controls. A class of drugs that inhibits PI3 kinase can restore normal levels of both protein synthesis and dendritic spine density in cultured hippocampal neurons from *Fmr1* KO mice. Tamping down PI3 kinase activity in fragile X neurons offers a different way to calm the overactive signalling in FXS⁹⁸.

Therapeutic consideration

The mGluR theory is focused on the group I mGluRs, mGluR1 and mGluR5, whose distribution differs in the brain. mGluR1 is mainly present in the cerebellum, whereas mGluR5 is predominantly expressed in the forebrain, including cerebral cortex, hippocampus, basal ganglia and amygdala^{99–100}. Therefore, negative modulators of mGluR5 will most likely target all of these brain regions. Currently, it is not known if all of these brain regions contribute to the FXS phenotype or whether negative mGluR5 modulators will act affect all these sites. Pharmacological studies will be necessary to investigate the efficacy and specificity of negative modulators of mGluR5 on different brain regions using the FXS mouse model.

Patients with FXS are treated with drugs to help with the disease symptoms or behavioural deficits, including hyperactivity and anxiety. Unfortunately, at present, a specific pharmaceutical treatment to correct underlying molecular abnormalities is not available. Nevertheless, attempts to discover new types of drugs to treat patients with FXS are underway, owing to improved insights into the molecular pathways involved in the pathogenesis of FXS. Testing new drugs in mouse models will be essential before they can be used in human clinical trials. Pretesting in mouse models is, however, no guarantee for success in clinical trials. Several new potentially therapeutic drugs have been tested directly in patients with FXS, such as acamprosate or memantine. If a drug is approved for use in other diseases and is considered to be safe for humans, the effects of this drug can be examined off-label in patients.

Several clinical trials have been conducted to study the effect of different types of drugs in patients with FXS that revealed preliminary data about safety and efficacy. However, to establish definitive efficacy, it is important to set up clinical trials that: (i) are randomized and placebo-controlled; (ii) are double-blind; (iii) include adequate number of patients; and (iv) utilise reliable objective readouts to determine therapeutic efficacy. Most clinical trials use distinct psychological questionnaires to determine the therapeutic efficacy of new treatments. However, improvement of behaviour is subjectively scored by caregivers and teachers. To guarantee objectivity, it is important to include other reliable and objective readouts to determine the efficacy of new treatments. A good candidate for such a test might be the PPI test (Box 3).

Box 3

Prepulse inhibition of acoustic startle response (PPI)

PPI is a behavioural test used in several neuropsychiatric disorders, including schizophrenia, Huntington's disease and FXS. PPI is thought to reflect sensorimotor gating mechanisms that are considered a fundamental component of information processing in the brain. PPI is mediated by brainstem circuits, but modulated by forebrain circuits^{104–105}. PPI is regulated by many neurotransmitter systems, including glutamate, GABA and NMDA¹⁰⁶.

In rodents, the startle response and PPI are usually measured as a response of the whole body. The magnetic distance measurement technique (MDMT) measures the eyeblink response with very high sensitivity after a startle pulse with or without a preceding pulse⁶⁰. Results of PPI measurements in *Fmr1* KO are not always consistent. Some studies have shown that *Fmr1* KO mice show enhanced PPI compared to wild-type mice^{107–108}, whereas recently, PPI was reduced compared to wild-type mice⁶⁰. The lack of consistency in PPI responses in *Fmr1* KO mice might result from methodological differences or perhaps differences in background strains of the mice.

In humans, PPI is usually measured using orbicularis oculi electromyogram (EMG), which records the contraction of the orbicularis oculi muscle in response to the short burst. Patients

with FXS have a reduced PPI compared to controls, illustrating abnormal sensorimotor gating^{106–107}. In addition to distinct psychological questionnaires, PPI can be used as an objective treatment outcome to test the efficacy of future novel therapeutic agents because PPI is well established in humans and measures activity in multiple neurotransmitter systems that are altered in Fmr1 KO mice.

In conclusion, in fewer than 20 years since the discovery of the underlying gene defect of FXS, targeted therapeutic strategies are being developed. It is likely that in the near future a treatment for FXS, based on intervention in mGluR or GABA receptor pathways or a combination of both, will become available and could significantly improve the lives of individuals with FXS.

Box 4

Outstanding questions

1. Are spine abnormalities a cause or consequence of intellectual disorders?
2. Can mouse models lead to treatment for patients with FXS?
3. What is the best read out for efficacy of treatment?
4. At what age should treatment for FXS begin?
5. Which aspects of the FXS will be responsive to therapeutic intervention?
6. What are the ethical issues concerning the treatment of adult patients with FXS to rescue cognitive impairment?

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Glossary box

Macroorchidism

abnormally large testes with unaffected fertility. Macroorchidism is a diagnostic feature for FXS. An orchidometer is used to determine if the testes are enlarged

Ampakine

a new class of compounds that influence the AMPA receptors and can enhance the functioning of the receptor by enhancing the excitatory synaptic transmission

Pervasive developmental disorders (PDDs)

delay in development of multiple basic functions including solicitation and communication. PDD is also referred as autism spectrum disorder (ASD). There are five types of PDDs: autism, Asperger's Syndrome, Rett syndrome, Childhood disintegrative disorder and PDD not otherwise specified (PDD-NOS)

Clinical Global Impressions-Improvement (CGI-I) scale

is commonly used to measure symptoms of severity, treatment response and treatment efficacy in clinical studies with patients with intellectual disability. The CGI-I is a seven points scale, which allows the clinician to assess the condition of the patients relative to the baseline state at the beginning of the intervention (1-very much improved to 7-very much worse)

Aberrant Behaviour Checklist-Community (ABC-C)

is a symptom checklist to assess behavioural problems of children or adults with intellectual disability at home, school or workplace. The list consists of five subscales: irritability and agitation, lethargy and social withdrawal, stereotypic behaviour, hyperactivity and noncompliance and inappropriate speech

Vineland Adaptive Behaviour Scales (VABS)

is a test that can measure a person's adaptive level of functioning. The tests assess personal self-sufficiency, such as walking, talking, getting dressed and preparing a meal. VABS can be used as an evaluation and a diagnostic tool for patients with intellectual disability

Repeatable Battery for the Assessment of Neuropsychological status (RBANS)

is a neuropsychological assessment that tests five domains: immediate memory, visuospatial memory, language, attention and delayed memory. It does not test executive function. It also measures decline in cognitive function and can measure change in a patient's neuropsychological status over time

Likert scale

is a psychometric scale. It is an ordered, one-dimensional scale from which respondents choose one option that best aligns with their view. There are typically between four and seven options

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Figure 1. The mGluR theory

(a) Stimulation of mGluR5 by glutamate induces local mRNA translation at the synapse. Local protein synthesis stimulates the internalization of AMPA receptors, which is essential for long-term synaptic plasticity. FMRP negatively regulates transcription and reduces the internalization of AMPA receptors. (b) By extrapolation from findings in *Fmr1* KO mice, neurons from patients with FXS have increased internalization of AMPA receptors in the absence of FMRP, which weakens the synapse.

Figure 2. The mTOR signalling pathway

Two signalling pathways downstream of mGluR5 affect translation the MEK-ERK-Mnk1 and the PI3K-mTOR pathway ¹⁰¹. Several FMRP target mRNAs (PP2A and PI3K) are members of second messenger cascades converging on ERK. ERK is a point of convergence of several signalling cascades. Phospho-ERK can trigger translational activities via Mnk1 and S6K. ERK phosphorylation is regulated by phosphatases such as PP2A. In *Fmr1* KO mice, PP2A is overactivated after mGluR5 stimulation, causing the rapid deactivation of ERK. Thus, misregulated ERK activation can be a first indicator for deficient translational regulation at the synapse. Activation of mTOR is one of the primary triggers for the initiation of cap-dependent translation via phosphorylation of 4E-BP and S6K. After stimulation of mGluR5, PI3K phosphorylates the membrane phospholipid PIP₂, converting it to PIP₃. PIP₃ recruits Akt to the membrane where it is phosphorylated and activated by PDK1. Akt activates mTOR by inhibiting TSC, a heterodimer of TSC1 and TSC2. TSC2 contains a GAP domain for the small G-protein Rheb. When TSC2 is phosphorylated, its GAP activity decreases, activating Rheb and mTOR. Subsequently, mTOR interacts with Raptor, which binds both 4E-BP and S6K. Active mTOR can phosphorylate S6K resulting in the phosphorylation of S6. In addition, active mTOR phosphorylates 4E-BP. Unphosphorylated 4E-BP binds tightly to eIF4e, whereas phosphorylated 4E-BP does not, thereby permitting eIF4F to form a translational initiation complex. Phosphorylation of S6 and 4E-BP finally results in mRNA translation. The role of FMRP in the mTOR pathway is indicated. Upon mGluR5 stimulation, PP2a activity is enhanced (<1 minute), which rapidly dephosphorylates FMRP so mRNAs are translated ³⁹. Extended mGluR5 activation (>5 minutes) suppresses PP2a activity and FMRP is phosphorylated by S6K. Phosphorylated FMRP represses the translation of target mRNAs ⁴⁰. Abbreviations: MEK, Mitogen-activated protein kinase kinase; ERK, Extracellular signal regulated kinase; Mnk1, Mitogen-activated protein kinase interacting serine/threonine kinase 1; PI3K, phosphoinositide-3 kinase; 4E-BP, 4E-binding protein; S6K, S6 kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol (3,4,5)-trisphosphate; PDK1, 3-phosphoinositide-dependent kinase 1; TSC, tuberous sclerosis complex; GAP, GTPase-activating protein

Figure 3. Therapeutic strategies for FXS

Schematic representation of a glutamate receptor excitatory and GABA receptor inhibitory synapse lacking FMRP. Drugs can interact with different types of neuronal receptors, which might rescue of the disturbed synaptic transmissions in FXS. Negative mGluR5 modulators, GABA_A agonists, GABA_B receptor agonists, NMDA receptor antagonists, positive AMPA receptor modulators are potential candidates to rescue the FXS phenotype by correcting altered synaptic plasticity. In addition, other therapeutic interventions, such as Lithium, minocycline and acamprosate also have a therapeutic rationale and might have beneficial effect on behavioural and cognitive phenotypes of FXS.

Table 1

Therapeutic intervention	Drug	Model	Results	References
mGluR5	MPEP	<i>dFmr1</i> fly	Rescue courtship behaviour Partial rescue mushroom body defects Memory defects	[58]
		<i>Fmr1</i> KO mice	Rescue audiogenic seizure and open field behaviour	[59]
			Rescue deficit in PPI of startle response	[60]
	Fenobam	<i>Fmr1</i> KO cultured hippocampal neurons	Rescue enhanced AMPA receptor internalization	[42]
		<i>Fmr1</i> KO mice	Rescue abnormal spine morphology cultured neurons	[60]
		FXS patients	Rescue abnormal spine morphology cultured neurons	[60]
GABA _A receptor	AFQ056, STX107 and RO4917523	unknown	Rescue deficit in PPI of startle response	Willemssen; personal communication
			6 out of 12 patients improve in PPI	[62]
			Not placebo controlled	http://www.clinicaltrials.gov www.seasidetherapeutics.com
GABA _B receptor	Nipocotic acid	<i>dFmr1</i> fly	Rescue lethality after increased levels of glutamate in food	[64]
	Creatinine	<i>dFmr1</i> fly	Rescue lethality after increased levels of glutamate in food	[64]
GABA _B receptor	Racemic baclofen	<i>Fmr1</i> KO mice	Rescue audiogenic seizures	[54]
NMDA receptor	Memantine	FXS patients	No significant effects	[84]
AMPA receptor	CX516	FXS patients	No significant effects	[74]
Other interventions	Lithium	<i>dFmr1</i> fly	Rescue courtship behavior Partial rescue mushroom body defects Memory defects	[58]
		<i>Fmr1</i> KO mice	Rescue audiogenic seizure and open field behaviour	[86]
		FXS patients	Improvement in behaviour, not in cognitive function Not placebo controlled	[89]
	Minocycline	<i>Fmr1</i> KO mice	Rescue abnormal spine morphology	[91]
		FXS patients	Rescue of anxiety Improvement in language, behaviour and attention Not placebo controlled	[92]

Therapeutic intervention	Drug	Model	Results	References
	Acamprosate	FXS patients	3 out of 3 patients with FXS improve in language Not placebo controlled	[96]
	Aripiprazole	FXS patients	8 out of 12 patients with FXS completed the study Improvement in behaviour and irritability Not placebo controlled	[96]
	PI3K inhibitors	<i>Fmr1</i> KO mice	Rescue of elevated protein levels and abnormal spine density	[98]