

Therapeutic Strategies in Fragile X Syndrome: Dysregulated mGluR Signaling and Beyond

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Fragile X syndrome (FXS) is an inherited neurodevelopmental disease caused by loss of function of the fragile X mental retardation protein (FMRP). In the absence of FMRP, signaling through group 1 metabotropic glutamate receptors is elevated and insensitive to stimulation, which may underlie many of the neurological and neuropsychiatric features of FXS. Treatment of FXS animal models with negative allosteric modulators of these receptors and preliminary clinical trials in human patients support the hypothesis that metabotropic glutamate receptor signaling is a valuable therapeutic target in FXS. However, recent research has also shown that FMRP may regulate diverse aspects of neuronal signaling downstream of several cell surface receptors, suggesting a possible new route to more direct disease-targeted therapies. Here, we summarize promising recent advances in basic research identifying and testing novel therapeutic strategies in FXS models, and evaluate their potential therapeutic benefits. We provide an overview of recent and ongoing clinical trials motivated by some of these findings, and discuss the challenges for both basic science and clinical applications in the continued development of effective disease mechanism-targeted therapies for FXS.

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INTRODUCTION

A majority of intellectual disabilities are idiopathic. Therefore, therapeutic strategies to treat these disorders are not usually designed to target the underlying pathological mechanisms. Rather, treatments are mostly limited to behavioral interventions or drug therapies that ameliorate comorbid symptoms, such as anxiety, aggression, or epilepsy (Davis *et al*, 2008; Depositorio-Cabacar and Zelleke, 2010; Brosnan and Healy, 2011). Fragile X Syndrome (FXS) is the most common inherited form of intellectual disability (ID) and the most common known monogenic cause of autism spectrum disorders (ASD). Research analyzing the underlying molecular mechanisms has led to the discovery of several promising targets for

disease-mitigating pharmacological therapies, which might also be useful in treating other cognitive disorders and ASDs (Wang *et al*, 2010b).

FXS is caused by a CGG trinucleotide expansion in the 5' untranslated region of the X-chromosomal *fragile X mental retardation 1* gene (*FMR1*) (Verkerk *et al*, 1991). This mutation leads to hypermethylation of the surrounding nucleotide sequences and transcriptional silencing of the *FMR1* gene (Pieretti *et al*, 1991). As a consequence, the encoded fragile X mental retardation protein (FMRP) is not expressed. The length of the trinucleotide expansion in *FMR1* determines disease expression; alleles containing ≤44 repeats are considered normal, gray zone alleles have 45–54 repeats, premutation alleles contain between 55 and 200 repeats, and alleles with ≥200 repeats are considered a full mutation (Maddalena *et al*, 2001). Only the full mutation causes silencing of the gene and thus FXS. However, premutation can lead to fragile X-associated premature ovarian insufficiency (FXPOI) in female carriers (Sherman, 2000) and an adult-onset neurodegenerative disease, the fragile X-associated tremor/ataxia syndrome (FXTAS) (Berry-Kravis *et al*, 2007). Apart from the usual CGG repeat expansion resulting in FXS, a missense

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mutation within FMRP was found in a severe case of FXS (De Boulle *et al*, 1993). Moreover, two recent studies identified several mutations within the open reading frame and the promoter region of the *FMR1* gene in patients with neurodevelopmental diseases and disorders similar to FXS, suggesting that dysregulation or dysfunction of FMRP is the cause of FXS-like symptoms (Collins *et al*, 2010a,b).

In this review, we summarize and evaluate advances in recent research elucidating the molecular functions of FMRP. We assess the potential of these findings to lead to therapeutic strategies for FXS or other disorders caused by *FMR1* loss-of-function mutations. We discuss current clinical trials targeting some of the pathological mechanisms caused by the absence of FMRP.

FRAGILE X CLINICAL PHENOTYPE

Individuals with a fragile X full mutation and FXS display characteristic physical features, ID, dysfunction in multiple behavioral domains, and specific medical problems. All aspects of the phenotype are more evident in males than females, as females express FMRP from the normal X chromosome, the amount of which depends on X inactivation ratios. Physical features and associated medical problems (Berry-Kravis *et al*, 2002; Hagerman *et al*, 2009) are variably present and include: macroorchidism (present in most adult males), prominent ears, macrocephaly, long face, high arched palate, hyperextensible joints, soft skin, recurrent otitis media and sinusitis, gastroesophageal reflux, strabismus and farsightedness (Maino *et al*, 1991), hyperphagia and obesity (Raspa *et al*, 2010; Utari *et al*, 2010), mitral valve prolapse and valvular insufficiency (particularly in adults), pes planus (flat feet), joint dislocations, scoliosis, disrupted sleep patterns (Kronk *et al*, 2010), and obstructive sleep apnea. Seizures occur in ~15% of males and 6–8% of females with FXS, and most commonly are partial complex (Musumeci *et al*, 1999; Berry-Kravis, 2002; Berry-Kravis *et al*, 2010), have onset between age 4 and 10 years, and resolve during childhood, although presence of seizures appears to be an indicator of an increased risk for autism in FXS (Berry-Kravis, 2002; Berry-Kravis *et al*, 2010). Although important to manage when present, the medical and physical problems are mild, and cognitive and behavioral impairments are the aspects of the phenotype that result in substantial functional limitations.

Cognitive/Developmental Phenotype

Young children with FXS are typically identified because of presentation with developmental (particularly speech) delays and/or hypotonia (Berry-Kravis *et al*, 2002). Males with a completely methylated full mutation usually display mild-to-moderate ID (average IQ 40–50, mental age 5 to 6 years for adults) with adaptive/achievement skills typically higher than IQ would predict (Berry-Kravis *et al*, 2002; Hagerman, 2002; Hagerman *et al*, 2009). IQ can be higher in

young boys and decline during mid-childhood in the absence of true regression, because of poor acquisition of complex processing skills relative to typically developing children (Berry-Kravis *et al*, 2002; Hagerman, 2002). Females with the full mutation often present with executive and non-verbal learning disabilities, although the average IQ is about 80 and ~25% have ID (de Vries *et al*, 1996). Males and females with FXS have a qualitatively similar cognitive profile that is quantitatively less severe in females, with strengths in receptive vocabulary, grammatical structure, visual memory, simultaneous processing, experiential learning, and imitation, and weaknesses in auditory processing, sequential processing, mathematical and quantity skills, abstraction, visuospatial and constructional ability, working memory, executive function and attention, and coordination and praxis (Berry-Kravis *et al*, 2002; Braden, 2002). Knowledge of the characteristic cognitive profile in FXS allows educational and therapy programming to be tailored to enhance learning by capitalizing on strengths and minimizing weaknesses (Braden, 2002).

Behavioral Phenotype

Males with FXS have characteristic behavioral features including hyperactivity, impulsivity, attention problems, generalized anxiety with phobias and multiple specific domains of anxiety (de Vries *et al*, 1996; Sullivan *et al*, 2007; Hagerman *et al*, 2009; Farzin *et al*, 2011), mood lability, and autistic features such as poor eye contact, shyness, self-talk, hand-flapping, hand-biting, hyperarousal to sensory stimuli, and substantial perseverative language and behavior (Berry-Kravis and Potanos, 2004; Hagerman *et al*, 2009; Wang *et al*, 2010b). A subset of these problems are common in females, including attention problems, impulsivity, shyness, selective mutism, specific phobias, social anxiety, and social deficits, often occurring even when the IQ is in the normal range (de Vries *et al*, 1996; Hagerman *et al*, 2009; Wang *et al*, 2010b). Individuals with FXS demonstrate an enhanced sympathetic response to sensory stimuli, as measured by electrodermal responses (Miller *et al*, 1999), heart rate variability (Boccia and Roberts, 2000), and pupillary responses (Farzin *et al*, 2009), and abnormal sensory gating in prepulse inhibition studies (Hessl *et al*, 2009). Aggression occurs in at least 30–50% of males, most often in adolescence (Hessl *et al*, 2008). Many boys with FXS (50–90%) display autistic behavioral patterns, particularly avoidant and anxious behaviors, and ~18–36% meet full criteria for Autistic Disorder, whereas ~43–67% of males and 20–23% of females with FXS have an ASD (Wang *et al*, 2010b). The patterns of social behavior in FXS often differ from typical autism even in individuals with FXS who meet criteria for ASD: individuals with FXS often have strong social interest but high levels of social anxiety. The behaviors in FXS often limit performance, and supportive treatment with behavioral therapy and medications targeted to the most severe behavioral symptoms are commonly utilized to improve functioning.

FMRP: A KEY REGULATOR OF NEURONAL FUNCTION

A major challenge in basic science is to understand how the loss of FMRP results in the behavioral and cognitive deficits in FXS. FMRP is an mRNA-binding protein that plays a role in various aspects of activity-dependent mRNA metabolism in neurons, such as mRNA transport (Dichtenberg *et al*, 2008; Estes *et al*, 2008), stability (Zalfa *et al*, 2007), and regulation of mRNA translation (see, eg, Zalfa *et al*, 2003; Muddashetty *et al*, 2007; Bechara *et al*, 2009). Here, we will focus on the role of FMRP in translational control, as this is the so far best-studied function of FMRP in RNA metabolism.

The molecular mechanisms whereby FMRP regulates basal and stimulus-induced translation are not fully understood and are controversially discussed in the field. FMRP was shown to repress translation of individual target mRNAs as well as general protein synthesis (Zalfa *et al*, 2003; Lu *et al*, 2004; Dolen *et al*, 2007; Muddashetty *et al*, 2007; Gross *et al*, 2010; Osterweil *et al*, 2010). On the other hand, recent work suggests that FMRP can also act as a translational activator for specific target mRNAs (Bechara *et al*, 2009; Fahling *et al*, 2009; Gross *et al*, 2011). FMRP has been proposed to regulate not only translation initiation (Zalfa *et al*, 2006; Napoli *et al*, 2008), but also translation elongation (Darnell *et al*, 2005). In addition, FMRP is associated with the RISC complex (Jin *et al*, 2004), implying a role in microRNA-mediated translational control. In fact, recent studies indicate that FMRP regulates translation of certain cargo mRNAs via specific microRNAs targeted to their 3'UTR sequences (Edbauer *et al*, 2010; Muddashetty *et al*, 2011). Future studies will have to address whether and how FMRP regulates different steps during mRNA translation and if this regulation varies for specific targets.

FMRP contains several RNA-binding domains: two KH domains, a C-terminal RGG box, and a more recently discovered N-terminal mRNA-binding motif (reviewed in Bassell and Warren, 2008). Although the RGG box was shown to associate with mRNAs carrying a G-quartet in their 3'UTR (Darnell *et al*, 2001), no physiological mRNA targets for the KH domains have been discovered so far. An *in vitro* study identified a tertiary mRNA structure, named the kissing complex, which binds to the second KH (KH2) domain of FMRP (Darnell *et al*, 2005). This study also suggested that the interaction of the KH2 domain with kissing complex harboring RNAs mediates the association of FMRP with polysomes. Of note, a missense mutation within the KH2 domain, which was found in a severe case of FXS (De Boulle *et al*, 1993), leads to loss of polysomal association of FMRP (Feng *et al*, 1997).

FMRP is involved in and affected by many activity-dependent neuronal mechanisms. *In vitro* screens have suggested that FMRP might associate with up to 4% of all mRNAs present in the brain (Brown *et al*, 2001; Miyashiro *et al*, 2003), and the identified mRNA targets encode proteins important for various cellular mechanisms, such as

cytoskeletal regulation, AMPA receptor trafficking, synaptic structure and composition, and synaptic signal transmission (Zalfa *et al*, 2003; Lu *et al*, 2004; Muddashetty *et al*, 2007; Schutt *et al*, 2009; Strumbos *et al*, 2010). FMRP is not only a multifaceted regulator of numerous target mRNAs, but is also itself activity-regulated at multiple steps, such as FMRP synthesis at synapses (Weiler *et al*, 1997), protein and mRNA localization in dendrites and at synapses (Antar *et al*, 2004), and posttranslational modifications and protein stability (reviewed in Bassell and Warren, 2008; De Rubeis and Bagni, 2010; Pfeiffer and Huber, 2009). In summary, these studies indicate that FMRP is tightly regulated in response to neuronal stimuli, and plays diverse roles in neuronal activity-dependent mechanisms of RNA metabolism. This complexity of functions and regulatory mechanisms, together with the variety of putative mRNA targets, suggests that FMRP is a key regulator of neuronal and synaptic development and plasticity, and might explain the various neurological problems associated with FXS. Here, we will focus on the emerging role of FMRP to regulate neuronal signal transduction and the promising therapeutic strategies that are arising from this function.

ANIMAL MODELS OF FXS

The knowledge about neuronal and molecular mechanisms that underlie FXS has been greatly advanced by the generation and analysis of animal models. The first FXS animal model developed was the *Fmr1* knockout (KO) mouse (The Dutch-Belgian Fragile *et al*, 1994), followed by *Drosophila* FXS models with a deleted or mutated *dfmr1* gene (Zhang *et al*, 2001; Morales *et al*, 2002), and zebrafish FXS models, in which *fmr1* expression was knocked down with antisense morpholinos or the *fmr1* gene was deleted by genetic knockout (Tucker *et al*, 2006; den Broeder *et al*, 2009). These transgenic or knockout animals do not carry the trinucleotide expansion, but do have functional deletions of FMRP. Although they are, therefore, not perfect models of the human disease, they have helped tremendously to reveal the molecular mechanisms underlying FXS. They recapitulate several symptoms observed in human patients, and the phenotypic presentation is comparably broad, with defects in neuronal development, dendritic spine morphology, synaptic plasticity, pain processing, and behavior.

The characterization of the FXS mouse model has been partially difficult because most phenotypes have rather small effect sizes and are sensitive to genetic background and experimental settings. Thus, several previously described molecular, cell biological, or behavioral phenotypes were difficult to reproduce in different laboratories, and are controversially discussed in the field (see below). Nevertheless, FXS animal models have been proven helpful to identify, develop, and test potential therapeutic strategies to treat FXS. Here, we will primarily focus on (1) robust phenotypes that are shown to be useful to test the effects of

specific drug treatments, and (2) recently discovered novel phenotypes that might become helpful assessment tools for FXS-targeted therapeutic strategies in the future.

Abnormal Dendritic Spine Morphology in FXS

Fragile X, like many other neurological diseases (Penzes *et al*, 2011), is characterized by abnormal dendritic spine morphology. Observations in post-mortem tissue from individuals with FXS and in the *Fmr1* KO mouse have shown that functional deletion of FMRP leads to increased density of filopodia-like and immature dendritic spines (Irwin *et al*, 2001, 2002), suggesting an important role of FMRP for dendritic spine development and maintenance. The analyzed parameters and the described dendritic spine abnormalities vary between different reports. They include *in vivo* and *in vitro* studies, as well as analyses of dendritic protrusion and filopodia density, dendritic spine classification, and dendritic arborization (see, eg, McKinney *et al*, 2005; de Vrij *et al*, 2008; Gross *et al*, 2010; Grossman *et al*, 2010). Some findings are contradictory; for example, several studies have reported significantly increased total dendritic spine density in cultured hippocampal *Fmr1* KO neurons as well as in cortex and olfactory bulb *in vivo* (Hayashi *et al*, 2007; Gross *et al*, 2010; Liu *et al*, 2011; Scotto-Lomassese *et al*, 2011), whereas other studies have detected increased numbers of filopodia, but no significant difference in total spine density in the cortex *in vivo* and in cultured hippocampal neurons (Irwin *et al*, 2002; de Vrij *et al*, 2008; Bilousova *et al*, 2009). Different experimental settings, such as age of the mice or neuronal cultures, might explain variations in the observed phenotypes. An earlier study, for example, has shown that the dendritic spine phenotype in *Fmr1* KO mice is age dependent (Nimchinsky *et al*, 2001). Although the exact parameters analyzed vary from study to study, abnormal dendritic spine morphology in *Fmr1* KO mice is a robust phenotype and was observed in many different laboratories (for a recent review of the dendritic spine phenotype, see Portera-Cailliau, 2011).

Several studies in *Fmr1* KO mice and *dfmr1* *Drosophila* mutants have demonstrated that FMRP is important for the development and activity-dependent plasticity of neuronal connections. These reports have provided considerable insight into the mechanism that might underlie abnormal synapse development and dendritic spine morphology in FXS. In *Drosophila*, FMRP is essential for synapse formation and experience-dependent axonal pruning during development (Gatto and Broadie, 2008; Tessier and Broadie, 2008). Studies in *Fmr1* KO mice have further revealed that FMRP regulates protein synthesis-dependent axon pruning, dendritic spine elimination, and actin-dependent stabilization of spines. In *Fmr1* KO mice, disruption of this regulation leads to abnormal rates of dendritic spine turnover, delayed stabilization of dendritic spines during development, and absence of experience-induced dendritic spine modulation (Pfeiffer and Huber, 2007; Li *et al*, 2009; Chen *et al*, 2010;

Cruz-Martin *et al*, 2010; Pan *et al*, 2010; Pfeiffer *et al*, 2010; Scotto-Lomassese *et al*, 2011).

The aberrant spine morphology in FXS might be due to loss of FMRP-dependent translational control of proteins necessary for synapse development or dendritic spine stability. One important group of FMRP-regulated proteins comprises cytoskeletal or scaffold proteins needed for synapse development and/or dendritic spine stability, such as MAP1B, PSD-95, or Shank (reviewed in Bassell and Warren, 2008). Dysregulation of these proteins in the absence of FMRP might contribute to the abnormal dendritic spine morphology in FXS. More recent studies reveal that the function of FMRP for microRNA-mediated translational control of specific targets such as NR2A and PSD95 is important for dendritic spine morphology (Edbauer *et al*, 2010; Muddashetty *et al*, 2011).

Furthermore, FMRP was proposed to regulate actin filament reorganization and stabilization mediated by the Rac/p21-activated kinase (PAK) signaling pathway (Chen *et al*, 2010). Interestingly, Chen *et al* (2010) also demonstrated that synaptic activity failed to induce the Rac/PAK pathway in *Fmr1* KO mice. This suggests that the absence of experience-dependent dynamic changes of spine morphology in FXS might be because of abnormal neuronal signal transduction regulating the actin cytoskeleton. Of note, a dominant-negative PAK transgene rescued increased dendritic spine density in *Fmr1* KO mice (Hayashi *et al*, 2007). However, it is not known whether the underlying mechanisms might involve loss of FMRP-mediated translational control of any Rac/PAK signaling components.

Most of the work analyzing the molecular mechanisms leading to abnormal spine morphology have been done *in vitro* in cultured neurons or *in vivo* in fixed tissue, which possibly limits their value. Only recently, studies have begun to analyze the function of FMRP for dendritic spine morphology *in vivo* in living mice (Cruz-Martin *et al*, 2010; Pan *et al*, 2010). These analyses revealed that dendritic spines are more transient and show delayed stabilization in *Fmr1* KO mice (reviewed in Portera-Cailliau, 2011). In the future, more studies are needed to test whether the mechanisms observed *in vitro* can be recapitulated *in vivo* in living animals. In addition, the identification of specific FMRP target mRNAs important for regulating dendritic spine morphology will provide further insight into the causes of aberrant dendritic spine development and dynamics in FXS.

Although the underlying molecular mechanisms are not fully understood yet, the dendritic spine phenotype in *Fmr1* KO mouse models has been proven to be an important readout to evaluate novel therapeutic strategies in FXS (Dolen *et al*, 2007; de Vrij *et al*, 2008; Bilousova *et al*, 2009; Gross *et al*, 2010; Liu *et al*, 2011).

Altered Neuronal Network Formation in FXS

Abnormal synapse development and morphology is expected to compromise synaptic function and might thus

contribute to the observed alterations in neuronal circuit formation in FXS animal models. Gatto and Broadie (2009) reported abnormalities in circadian clock circuits in *dfmr1* mutant *Drosophila*, which were due to aberrant synaptic architecture and could be rescued by temporally controlled overexpression of dFMRP. In *Fmr1* KO mice, dysregulated neuronal connectivity in the barrel cortex (Bureau et al, 2008) leads to defective glutamatergic synapse maturation, delayed and aberrant formation of sensory maps, and altered synaptic plasticity during the critical period (Harlow et al, 2010). In general, loss of FMRP seems to lead to altered network synchrony and hyperexcitable neuronal networks (Chuang et al, 2005; Gibson et al, 2008).

The underlying mechanisms of network synchrony abnormalities are only poorly understood so far. Evidence from FXS *Drosophila* and mouse models suggests that calcium signaling is disturbed in the absence of FMRP, which could partially account for defects in neuronal network formation (Meredith et al, 2007; Tessier and Broadie, 2008). Furthermore, a recent report proposed a role for FMRP in retinoic acid-mediated homeostatic plasticity, an important signaling pathway regulating network activity (Soden and Chen, 2010). This suggests that dysregulated neuronal signal transduction contributes to altered network activity in FXS. Dysregulation of several FMRP targets might also contribute to aberrant network plasticity in FXS. Arc/Arg3.1, which was suggested to be an FMRP target (Zalfa et al, 2003; Krueger et al, 2011), was shown to be involved in neuronal network homeostasis (Shepherd et al, 2006; Peebles et al, 2010; Béïque et al, 2011). Moreover, lately, a few studies have reported that FMRP regulates potassium channels, which might also contribute to abnormal network activity in FXS. Expression and/or function of three different potassium channels have been suggested to be altered in the absence of FMRP: the sodium-activated potassium channel Slack (Brown et al, 2010), and the voltage-gated potassium channels Kv3.1b (Strumbos et al, 2010) and Kv4.2 (Gross et al, 2011).

Taken together, several studies have demonstrated that absence of FMRP leads to aberrant neuronal network homeostasis in animal models. Additional studies are needed to investigate if and how FMRP directly influences neuronal network synchrony, for example, by regulation of the function or protein expression of specific targets.

Altered Synaptic Plasticity in *Fmr1* KO Mice

The dysfunctions in neuronal network formation might contribute to dysregulated synaptic plasticity observed in FXS. The most prominent and best-studied example is hippocampal metabotropic glutamate receptor 1/5 (mGlu_{1/5})-dependent synaptic long-term depression (LTD), which is exaggerated and, in contrast to wild type, protein synthesis independent in *Fmr1* KO hippocampus (Huber et al, 2002; Nosyreva and Huber, 2006; Hou et al, 2006; further discussed below). Several studies have also shown impaired long-term potentiation (LTP) in different cortical areas of

Fmr1 KO mice (Li et al, 2002; Zhao et al, 2005; Desai et al, 2006). In contrast, LTP appeared to be normal in the *Fmr1* KO hippocampus (Paradee et al, 1999; Li et al, 2002; Larson et al, 2005). However, a defect in hippocampal LTP could be detected at lower levels of stimulation (Lauterborn et al, 2007), which was in line with another study showing that spike-timing-dependent LTP in the *Fmr1* KO cortex was normal after a strong stimulus, but impaired when using a threshold induction paradigm (Meredith et al, 2007). Together, these findings suggest that in the absence of FMRP, neurons are capable of developing LTP, but require a stronger stimulus than wild-type neurons. In addition, more recently, specific mGlu_{1/5}-dependent forms of LTP and LTP priming were shown to be altered in the *Fmr1* KO hippocampus (Shang et al, 2009; Auerbach and Bear, 2010; further discussed below). Apart from hippocampus and cortex, impaired long-term synaptic plasticity has also been shown in the amygdala (reviewed in Suvrathan and Chattarji, 2011) and the cerebellum of *Fmr1* KO mice (reviewed in Huber, 2006).

In summary, loss of FMRP leads to impairments in several forms of long-term synaptic plasticity in different brain areas in *Fmr1* KO mice. Future studies analyzing, for example, which phases of LTP are affected in the *Fmr1* KO brain might identify the signal transduction pathways involved in defective synaptic plasticity in *Fmr1* KO mice, and may help to develop therapeutic strategies to treat cognitive impairments in patients with FXS.

The Behavioral Phenotype of *Fmr1* Mouse Models

FXS is a complex disorder and patients suffer from a variety of neurological problems, such as intellectual disabilities, epilepsy, and autism. The behavioral characterization of the FXS mouse model has covered several aspects of this disease pattern including: (1) cognitive performance, (2) seizure susceptibility and general neuronal activity, (3) sensitivity to sensory stimuli, and (4) socioemotional behavior. In general, the evaluation of behavioral phenotypes in *Fmr1* KO mice has been challenging because the observed defects are rather subtle and seem to strongly depend on genetic background, age, and experimental settings (reviewed, eg, in Bernardet and Crusio, 2006; Bhogal and Jongens, 2010). Initial studies using 'classic' hippocampus-dependent learning tasks, such as Morris Water Maze and fear conditioning, revealed no or very subtle defects in the *Fmr1* KO mice (The Dutch-Belgian Fragile et al, 1994; Paradee et al, 1999; Van Dam et al, 2000). Interestingly, a recent report has shown that prefrontal cortex-dependent forms of learning are altered in *Fmr1* KO mice (Krueger et al, 2011), and patients with FXS are specifically impaired in several prefrontal cortex-dependent cognitive skills (Hoeft et al, 2007; Reiss and Hall, 2007; Mercaldo et al, 2009). This suggests that behavioral paradigms testing prefrontal cortical function in *Fmr1* KO mice might be a better tool to assess therapeutic strategies for their potential to rescue cognitive impairment in FXS (Krueger et al, 2011).

Box 1 FXS, PI3K/mTOR Signaling, and Autism

Fragile X syndrome is the most frequent monogenetic form of autism, and evidence is emerging that *Fmr1* KO mice represent a suitable animal model to study autism. Paradigms that assess stereotypic behaviors and socioemotional defects, such as, marble burying and the social partition test, respectively, might thus be useful to assess novel drugs for their effectiveness to ameliorate the socioemotional problems and autistic phenotypes in FXS (Bernardet and Crusio, 2006; Silverman et al, 2010; Ey et al, 2011). Results need to be evaluated carefully though, as a recent study suggests that genetic background differences strongly influence these autistic-like phenotypes in *Fmr1* KO mice (Spencer et al, 2011). Autism spectrum disorders (ASDs) are a complex and diverse group of disorders, which can be caused by different factors such as gene mutations, epigenetics, and environmental factors (reviewed in van de Lagemaat and Grant, 2010). Over the past years, evidence is emerging that the PI3K/mTOR signaling pathway is frequently linked to autism, via both inherited and exogenous mechanisms, suggesting that dysregulated PI3K/mTOR signaling might be a common mechanism in many ASDs (reviewed in Levitt and Campbell, 2009). The observation that PI3K/mTOR signaling in FXS is dysregulated (Ronesi and Huber, 2008; Gross et al, 2010; Sharma et al, 2010) might therefore provide another interesting link between FXS and autism. Several single-gene mutations associated with autism affect components of the PI3K/mTOR signaling complex, for example, mutations or deletions in *tuberous sclerosis complex 1/2* (*TSC1/2*) (Wiznitzer, 2004), and *phosphatase and tensin homolog on chromosome ten* (*PTEN*) (Butler et al, 2005; Redfern et al, 2010). Furthermore, an extensive study of CNVs in autistic individuals has identified PI3K/mTOR pathway-related genes as 'hotspots' for CNVs in autism (Cusco et al, 2009). Moreover, several neurotransmitter-coupled pathways that have been linked to ASDs also regulate PI3K/mTOR activity, such as GABA-, BDNF-, and serotonin-dependent signaling (Koishi et al, 2006; Eagleson et al, 2010; Sheikh et al, 2010). Of note, a major cellular function of PI3K/mTOR signaling is the regulation of protein synthesis, which was suggested to be defective in many ASDs (Kelleher and Bear, 2008). In the future, studies to evaluate the PI3K/mTOR signaling pathway as potential therapeutic target might thus be beneficial for several ASDs of diverse etiologies.

The most promising behavioral assays to evaluate new rescue strategies in FXS mouse models are those that are targeted on neuronal hyperexcitability and have correlates in human patients, such as epilepsy and hyperactivity. One of the most robust assays tests the susceptibility to audiogenic seizures. Higher susceptibility to audiogenic seizures is reliably observed in *Fmr1* KO mice of various genetic backgrounds and has become very useful to evaluate potential therapeutic strategies (Musumeci et al, 2000; Yan et al, 2004, 2005; Dolen et al, 2007; Min et al, 2009). Recently, a kindling paradigm similarly revealed differences in seizure susceptibility in wild-type and *Fmr1* KO mice (Qiu et al, 2009). If reproducible, this seizure paradigm might become an additional tool to assess hyperexcitability in the future. Apart from seizure activity, altered open field activity in mice was reproduced by several laboratories. Although this phenotype is less robust and seems to vary with age and experimental settings (Bernardet and Crusio, 2006), it was used successfully to test potential therapeutic targets for FXS (Spencer et al, 2005; Yan et al, 2005; Yuskaitis et al, 2010). Additionally, behaviors that phenotype the hypersensitivity to sensory stimuli observed in FXS patients, such as altered auditory startle responses and impaired prepulse inhibition, have been reproduced in several laboratories and used to show the efficacy of drug treatments (reviewed in Bernardet and Crusio, 2006;

Brodin, 2008). Of interest, *Fmr1* KO mice display autistic-like behaviors and have been suggested as valuable mouse model for autism (Bernardet and Crusio, 2006; discussed in Box 1).

In the future, behavioral assays in *Fmr1* KO mice will have to be better standardized for experimental paradigms, age, and genetic background to provide a valuable and reproducible tool for the evaluation of novel therapeutic strategies in FXS.

The characterization of FXS animal models suggests that FMRP regulates diverse neuronal functions and mechanisms. As detailed below, many of the described neuronal defects can be attributed to the function of FMRP as a major regulator of neuronal signal transduction controlling protein synthesis at the synapse. Therefore, dysregulated signaling pathways in the absence of FMRP are promising targets for therapeutic interventions in patients with FXS.

THE mGluR THEORY: THERAPEUTIC VALUE AND LIMITATIONS

Increased and Protein Synthesis-Independent mGlu_{1/5} LTD in FXS

In 2002, a seminal study by Huber et al (2002) reported that Glu_{1/5}-mediated LTD, a specific protein synthesis-dependent form of synaptic plasticity in the hippocampus, is elevated in *Fmr1* KO mice. This discovery motivated the formulation of the mGluR theory of FXS, which posits that dysregulated mGlu_{1/5}-mediated protein synthesis-dependent forms of synaptic plasticity contribute to the pathology of FXS (Bear et al, 2004). Hence, Bear et al (2004) proposed that mGlu_{1/5} signaling might be a promising therapeutic target for FXS. Follow-up studies further demonstrated that, in contrast to wild-type, mGlu_{1/5} LTD is protein synthesis-independent in *Fmr1* KO mice (Nosyreva and Huber, 2006; Hou et al, 2006), thus corroborating the assumption that a major regulator of neuronal protein synthesis is lost in FXS.

A crucial molecular mechanism underlying induction of mGlu_{1/5}-mediated LTD is the protein synthesis-dependent increase of GluA endocytosis (for a detailed review, see Gladding et al, 2009). This mechanism appears to be compromised in FXS, because reduced levels of FMRP were shown to lead to increased mGlu₅-mediated GluA endocytosis in rat hippocampal neurons (Nakamoto et al, 2007). The mGlu_{1/5}-dependent translational induction of two FMRP targets, Arc/Arg3.1 and MAP1B, which were suggested to be involved in activity-regulated GluA endocytosis in hippocampal neurons (Davidkova and Carroll, 2007; Waung et al, 2008), is absent in *Fmr1* KO mice (Hou et al, 2006; Park et al, 2008). Waung and Huber (2009) thus hypothesized that in the absence of the translational repressor FMRP, several 'LTD-proteins' are already present in excess at the synapse before LTD-inducing stimuli and could contribute to the exaggerated LTD phenotype. This hypothesis is in line with previous studies suggesting increased basal protein expression of MAP1B in the

hippocampus and Arc/Arg3.1 in total brain fractions from *Fmr1* KO mice (Zalfa et al, 2003; Lu et al, 2004). However, in contrast to Zalfa et al (2003), another study reported reduced Arc/Arg3.1 protein levels in the orbital frontal cortex and medial prefrontal cortex of FXS mice (Krueger et al, 2011). Future experiments are needed to analyze whether FMRP regulates specific target mRNAs differently depending on the brain region.

Taken together, these studies suggest that the role of FMRP in regulating mGlu_{1/5}-induced protein synthesis might underlie the exaggerated LTD phenotype in FXS mice. In the future, further identification and analysis of FMRP target mRNAs that have a function for mGlu_{1/5} LTD will help to fully understand the mechanisms leading to dysregulated LTD in FXS.

Other mGlu_{1/5}-Dependent Mechanisms in FXS

In support of the mGluR theory, several studies have demonstrated that mGlu_{1/5}-mediated neuronal and synaptic protein synthesis are elevated and stimulus-insensitive in *Fmr1* KO cortex and hippocampus (Dolen et al, 2007; Muddashetty et al, 2007; Gross et al, 2010; Osterweil et al, 2010). Subsequent work also suggested that apart from hippocampal LTD, additional mGlu_{1/5}-dependent neuronal mechanisms are dysregulated in the FXS brain. In the hippocampus, deletion of FMRP was shown to lead to prolonged epileptiform discharges caused by enhanced sensitivity of mGlu_{1/5} to synaptically released glutamate (Chuang et al, 2005). Loss of FMRP also leads to changes in mGlu_{1/5}-mediated endocannabinoid (eCB) synthesis and release in the hippocampus (Zhang and Alger, 2010). Several other brain regions display altered mGlu_{1/5}-dependent signaling. mGlu_{1/5} signaling was shown to be dysregulated in the anterior cingulate cortex of *Fmr1* KO mice (Wang et al, 2008a, 2009). Moreover, Harlow et al (2010) demonstrated impaired synaptic plasticity in the barrel cortex in *Fmr1* KO mice during the critical period, a process that was shown to involve FMRP and mGlu_{1/5} activity (Todd et al, 2003). Another study reported aberrant mGlu_{1/5}-dependent neuronal plasticity in the amygdala of *Fmr1* KO mice (Suvrathan et al, 2010). Furthermore, a specific, mGlu_{1/5}- and protein synthesis-dependent form of hippocampal LTP was shown to be dysregulated in *Fmr1* KO mice (Shang et al, 2009), and a recent study demonstrated that mGlu_{1/5}-mediated priming of hippocampal LTP is, in contrast to wild-type, protein synthesis-independent in *Fmr1* KO mice (Auerbach and Bear, 2010). Apart from LTD in the hippocampus, mGlu_{1/5}-dependent LTD was also shown to be exaggerated in the cerebellum in FXS mice (Koekkoek et al, 2005; Huber, 2006). The defects in mGlu_{1/5}-dependent pathways in these different brain areas support speculations that dysregulated mGlu_{1/5} signaling in patients with FXS might not only underlie cognitive impairments, but also epilepsy (epileptiform discharges in the hippocampus), hypersensitivity to sensory stimuli (barrel cortex), anxiety (amygdala), and motor

learning defects (cerebellum). Besides, Price et al (2007) showed that mGlu_{1/5}-dependent nociception in the spinal cord of *Fmr1* KO mice is altered and proposed that this may contribute to self-injurious behavior and altered perception of sensory stimuli and pain in patients. The correlation of phenotypes in animal models of FXS with those in humans is in most parts still hypothetical, but nevertheless these studies support the hypothesis that therapies reducing mGlu_{1/5} signaling might ameliorate several different symptoms in patients.

In line with these findings of dysregulated mGlu_{1/5} signaling in numerous paradigms, several rescue studies in mouse (Yan et al, 2005; Dolen et al, 2007) and *Drosophila* (McBride et al, 2005) models provided compelling support of the mGluR theory. These studies showed that both pharmacologic and genetic reduction of mGlu₅ signaling could ameliorate or reverse FXS-associated phenotypes *in vivo* and *in vitro* (reviewed in Levenet et al, 2010).

The underlying mechanisms of increased and stimulus-insensitive mGlu_{1/5} signaling are still being explored, and findings are partially controversial. In general, so far, there is no compelling evidence that FMRP directly regulates mGlu_{1/5} receptor expression or function. A study by Giuffrida et al (2005) showed that in *Fmr1* KO mice, less mGlu_{1/5} receptors are associated with the scaffolding protein Homer. Homer links mGlu_{1/5} to the phosphoinositide-3 kinase (PI3K) enhancer PIKE and the catalytic PI3K subunit p110. This is an alternative pathway to activate PI3K signaling downstream of mGlu_{1/5}, apart from activation via heterotrimeric G-proteins (Kurosu et al, 1997; Rong et al, 2003) (also see Figure 1). Interestingly, Ronesi and Huber (2008) reported that, in contrast to wild-type, neither mGlu₅–Homer interactions nor mGlu_{1/5}-mediated activation of PI3K signaling are necessary to elicit LTD in *Fmr1* KO mice. Although induction of PI3K signaling was impaired in *Fmr1* KO hippocampus, no elevated basal PI3K downstream signaling could be detected. This finding is in line with a report from Osterweil et al (2010), but in contrast to two other recent studies showing excess basal PI3K activity and downstream signaling (Gross et al, 2010; Sharma et al, 2010). These apparent contradictions might be because of differences in the examined tissue, but further analyses are needed to resolve these discrepancies and understand the molecular mechanisms of impaired mGlu_{1/5}–Homer–PI3K signaling. In summary, the above discussed studies suggest that mGlu_{1/5} might be uncoupled from downstream signaling in FXS, which could, for example, be explainable by a role of FMRP for regulating signal transduction.

Taken together, although the underlying molecular mechanisms are still under investigation, it has become evident from several studies that FMRP plays an essential role for stimulus-induced mGlu_{1/5} signaling. The successful validation of the mGluR theory in different animal models and paradigms further substantiates the value of mGlu_{1/5} as a therapeutic target in FXS, and has led to the development of mGlu₅-specific antagonists and the initiation of clinical trials targeting mGlu₅ signaling in FXS.

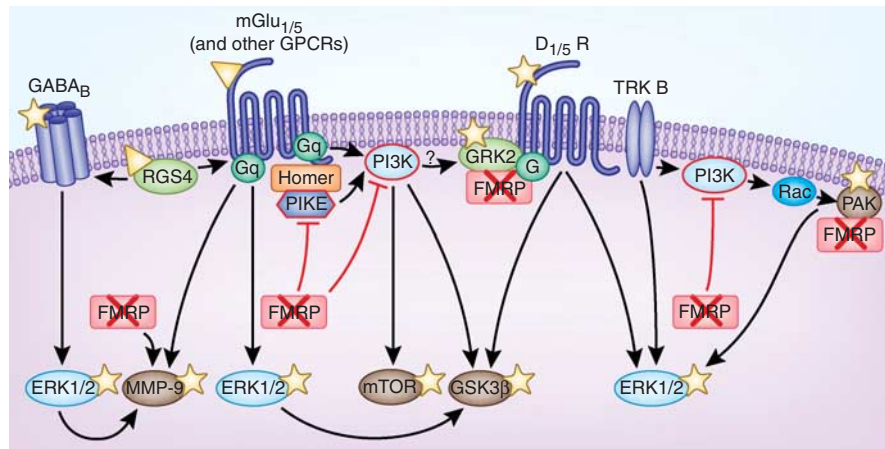


Figure 1. Dysregulated signal transduction as therapeutic target in FXS. Shown are signaling pathways and molecules that have proven to be promising targets for therapeutic treatment based on preliminary clinical trials and/or studies in FXS animal models. Targets can be divided into three major groups: (1) membrane receptors such as GABA, mGlu_{1/5}, and dopamine D_{1/5} receptors and their regulator RGS4; (2) the central intracellular signaling molecules ERK1/2 and PI3K; and (3) downstream targets such as MMP-9, mTOR, GSK3 β , GRK2, and PAK. Although genetic and pharmacologic studies suggest that these proteins might be promising therapeutic targets, the underlying mechanisms are mostly elusive, with a few exceptions: FMRP was shown to directly regulate PI3K and PIKE mRNA translation, protein expression, and enzymatic activity (indicated by red outlines) (Gross *et al*, 2010; Sharma *et al*, 2010). Furthermore, FMRP associates with PAK and GRK2 protein, but the functional consequences of these interactions are unknown (Hayashi *et al*, 2007; Wang *et al*, 2008b). Some potential therapeutic targets show dysregulated expression (MMP-9, GABA receptors), subcellular localization (PAK and GRK2), and/or phosphorylation (ERK1/2, GSK3 β , PAK, GRK2, mTOR) in the absence of FMRP (highlighted with yellow asterisks), but the detailed mechanisms are unknown and future studies will have to show whether these are the direct effects of loss of FMRP or are caused by dysregulated signaling through other pathways. Yellow triangles mark mGlu receptors and the regulator of G-protein signaling RGS4, which were shown to be promising therapeutic targets, but do not seem to be directly altered by loss of FMRP. Black arrows indicate how upstream membrane receptors activate and/or regulate some of the downstream targets, illustrating crosstalk between pathways and putative shared dysregulated downstream mechanisms: MMP-9 was suggested to be regulated by mGlu_{1/5} signaling (Bilousova *et al*, 2009) and by GABAergic signaling via an ERK-dependent mechanism (Miao *et al*, 2010). ERK1/2 and PI3K are regulated by mGlu_{1/5} (Banko *et al*, 2006) and by TrkB signaling (Yoshii and Constantine-Paton, 2010). ERK1/2 was shown to signal downstream of D_{1/5} (Beaulieu and Gainetdinov, 2011). A study suggests that PI3K signaling might affect GRK2 activity (Banday *et al*, 2007), which might contribute to the crosstalk between mGlu_{1/5} and D_{1/5} (Deng *et al*, 2010). GRK2 was suggested to be involved in dysregulated signaling through D1 receptors in *Fmr1* KO mice (Wang *et al*, 2008b). PI3K can regulate PAK activity via Rac and ERK1/2 itself was shown to be regulated by Rac/PAK signaling (Lim *et al*, 1996; Welch *et al*, 2003). GSK3 β activity is affected by both ERK1/2 and PI3K, downstream of mGlu_{1/5} and D_{1/5} (Lebel *et al*, 2009; Min *et al*, 2009).

Altered Signaling of Other Membrane Receptors

Dysregulated signaling through mGlu_{1/5} was one of the first molecular mechanisms shown to contribute to the pathology of FXS. Later on, several other neurotransmitter receptor-mediated signal transduction pathways regulating neuronal protein synthesis have been demonstrated to be affected in FXS. Aberrant signaling in the absence of FMRP was shown to occur through (1) other Gq protein-coupled receptors such as muscarinic acetylcholine receptors (Volk *et al*, 2007), (2) the dopamine D_{1/5} receptors (Wang *et al*, 2008b, 2010a), and (3) tyrosine kinase receptor B (TrkB) (Osterweil *et al*, 2010). Of note, these signaling pathways employ similar molecular mechanisms like mGlu_{1/5}-induced LTD. Activation of signaling through both Gq-coupled receptors and dopamine D1 receptors induces protein synthesis-dependent GluA receptor endocytosis (Volk *et al*, 2007; Wang *et al*, 2010a). Likewise, TrkB signaling has also been shown to stimulate protein synthesis and regulate GluA receptor surface expression (Caldeira *et al*, 2007; Minichiello, 2009). Similar to mGlu_{1/5} signaling in FXS, altered signal transduction mediated by these receptors is thus believed to be caused by the lack of activity-regulated translational control in the absence of FMRP.

Several studies suggested modulation of GABAergic inhibitory signaling as an alternative or additional therapeutic strategy in FXS: *Fmr1* KO mice show impaired GABAergic inhibition in several different brain areas (Centonze *et al*, 2007; Curia *et al*, 2009), which is in line with the notion that the absence of FMRP converts neurons into a 'hyperexcitable' state. Impaired GABAergic signaling in FXS might partially be caused by decreased expression of GABA receptors in these brain areas (El Idrissi *et al*, 2005; D'Hulst *et al*, 2006; Adusei *et al*, 2010). On the other hand, it could be a secondary effect of dysregulated mGlu_{1/5}-dependent signaling, because these two receptors were shown to crosstalk in several different brain areas (Hirono *et al*, 2001; Deng *et al*, 2010; Kolaj and Renaud, 2010). The exact mechanisms of reduced GABA-mediated signaling in FXS are currently unclear; nevertheless, results from animal studies and clinical trials suggest that GABA agonists are suitable to treat FXS-associated symptoms (Chang *et al*, 2008; Heulens *et al*, 2010; Olmos-Serrano *et al*, 2010).

Overall, these findings have two important implications: first, they have revealed additional promising therapeutic targets in FXS, and second, they provide important mechanistic insights suggesting that FMRP might regulate

a downstream mechanism shared by several signal transduction pathways involved in stimulus-dependent protein synthesis and neuronal plasticity.

FXS EMERGES AS A DISEASE OF DYSREGULATED SIGNALING

In light of these and other recent findings, FXS emerges from being a disease of dysregulated mGlu_{1/5} signaling to a disease of dysregulated general neuronal signaling upstream of protein synthesis (summarized in Figure 1). In support of this hypothesis, FMRP was shown to regulate general stimulus-induced neuronal and synaptic protein synthesis (Weiler *et al*, 2004; Qin *et al*, 2005; Dolen *et al*, 2007; Muddashetty *et al*, 2007; Gross *et al*, 2010; Osterweil *et al*, 2010). A study by Napoli *et al* (2008) suggested that FMRP directly regulates translation initiation by interaction with CYFIP1, which binds to and inhibits the translation initiation factor eIF4E. In addition, one could speculate that specific target mRNAs of FMRP might encode translation factors or intracellular signal transducers that are important for activity-dependent protein synthesis. As discussed below, recent studies support the hypothesis that FMRP might regulate key signaling molecules involved in translation initiation.

Targeting Downstream Signaling Molecules in FXS

In support of the hypothesis that FMRP has a crucial function for neuronal signal transduction, the genetic or pharmacologic inhibition of several molecules downstream of neurotransmitter-induced signaling have been shown to reverse relevant phenotypes in FXS animal models (Figure 1). Those proteins included not only glycogen-synthase kinase 3 β (GSK3 β), metalloproteinase 9 (MMP-9), PAK, and regulator of G-protein signaling 4 (RGS4), but also key cellular signal transducers such as extracellular signal-regulated kinase 1/2 (ERK1/2) and PI3K.

Fmr1 KO mice display elevated and dysregulated GSK3 β activity, a downstream signaling target of mGlu_{1/5} (Min *et al*, 2009; Yuskaitis *et al*, 2010). Several studies demonstrated that lithium, which among other things antagonizes GSK3 α and GSK3 β activity (Berridge *et al*, 1989; De Sarno *et al*, 2002), could rescue dysregulated GSK3 β activation, aberrant dendritic spine morphology, and several behavioral phenotypes in *Fmr1* KO mice (Liu *et al*, 2011; Mines *et al*, 2010; Yuskaitis *et al*, 2010), as well as cognitive impairments in a *Drosophila* model of FXS (Choi *et al*, 2010). Pharmacologic studies in *Fmr1* KO mice also suggested that the MMP-9 inhibitor minocycline might be a useful drug to ameliorate FXS symptoms (Bilousova *et al*, 2009). MMP-9 protein levels were shown to be increased by mGlu_{1/5} stimulation in wild-type mice and under basal conditions in *Fmr1* KO mice (Bilousova *et al*, 2009). Future studies will have to show whether MMP-9 is a direct target of FMRP or whether the dysregulated expression of MMP-9, and thus the therapeutic benefits of minocycline, are a

treatable indirect effect of dysregulated signaling downstream of mGlu_{1/5} receptors in FXS. MMP-9 was also shown to be regulated by GABA signaling via an ERK1/2-dependent mechanism (Tyagi *et al*, 2009). Thus, MMP-9 antagonists might improve dysregulated signaling through several pathways. Both lithium and minocycline have already been tested in clinical studies in patients with FXS (Berry-Kravis *et al*, 2008a; Paribello *et al*, 2010, see below).

Genetic rescue studies in *Fmr1* KO mice suggest that PAK and RGS4 might be putative therapeutic targets in FXS, but need to be confirmed in independent studies. Postnatal expression of a dominant-negative form of PAK reversed increased dendritic spine density and several behavioral phenotypes in *Fmr1* KO mice (Hayashi *et al*, 2007), motivating the development of PAK-specific inhibitors (www.afraxis.com). Furthermore, deletion of RGS4, a protein involved in downstream signaling of G-coupled receptors, such as GABA and mGlu receptors, in *Fmr1* KO mice could rescue altered social behavior, increased body weight, and aberrant synaptic translation (Pacey *et al*, 2011). However, other *Fmr1* KO phenotypes, such as hyperactivity and macroorchidism, were not affected by knockout of RGS4, suggesting that these might be regulated by different or additional molecular mechanisms.

In summary, these studies show that the loss of FMRP leads to dysregulation of multiple signaling pathways, and extend the list of potentially valuable targets for therapeutic treatments in patients with FXS. These findings also suggest that FMRP might control shared signaling molecules that are key players to regulate neurotransmitter-mediated signaling and protein synthesis. The two major pathways regulating neurotransmitter-induced protein synthesis in neurons are the ERK1/2 and the PI3K/mTOR (mammalian target of rapamycin) pathways (Banko *et al*, 2006; Nagai *et al*, 2007; Schicknick *et al*, 2008; Santos *et al*, 2010; Zhou *et al*, 2010). Both pathways also play crucial roles for intracellular signaling, and many of the above discussed dysregulated neurotransmitter-dependent signaling mechanisms in FXS are regulated or mediated via ERK1/2 and/or PI3K signaling (Figure 1). Therefore, antagonizing central downstream signaling molecules like ERK1/2 or PI3K might correct a broad variety of different FXS-associated symptoms caused by any of these signaling defects. ERK1/2 or PI3K antagonists could offer an alternative approach to therapeutic strategies targeting an upstream neurotransmitter receptor or downstream signaling molecules that are restricted to specific pathways.

PI3K and ERK Signaling as Therapeutic Targets

In support of the hypothesis that ERK1/2 and PI3K might be promising therapeutic targets in FXS, several recent studies suggest a function for FMRP in controlling the activity of ERK1/2 and PI3K/mTOR signaling pathways. Currently, the findings regarding ERK1/2 and PI3K/mTOR signaling in FXS are controversial. Several studies report deficient mGlu_{1/5}-mediated ERK1/2 activation (Hou *et al*, 2006;

Kim *et al*, 2008; Weng *et al*, 2008; Osterweil *et al*, 2010), whereas others have shown that the PI3K/mTOR signaling response to mGlu_{1/5} stimulation is lost (Ronesi and Huber, 2008; Gross *et al*, 2010; Sharma *et al*, 2010). Most of these studies analyze phosphorylation of downstream targets to evaluate basal and stimulus-induced activity of PI3K and ERK1/2. In general, these results have to be evaluated carefully, as ERK1/2 and PI3K/mTOR signaling pathways are not separate entities, but rather exist in a signaling network, implying that dysregulation of one of these pathways in FXS might result in aberrant signaling through the other pathway. Of note, a direct comparison of basal enzymatic activity of ERK1/2 and PI3K showed excess PI3K activity, but normal ERK1/2 activity in *Fmr1* KO mice (Gross *et al*, 2010). As a possible mechanism underlying excess PI3K activity, FMRP was shown to regulate protein expression of two PI3K activity-modulating enzymes, the catalytic PI3K subunit p110 β and the PI3K enhancer PIKE (Gross *et al*, 2010; Sharma *et al*, 2010), whereas the mechanisms underlying dysregulated ERK1/2 signaling remain elusive. However, both ERK1/2 and PI3K antagonists were shown to rescue phenotypes in FXS mice: intraperitoneal injection with an ERK1/2 inhibitor prevented audiogenic seizures in *Fmr1* KO mice (Osterweil *et al*, 2010), and PI3K inhibitors reduced excess dendritic spine density and aberrant GluA receptor endocytosis in *Fmr1* KO hippocampal neurons (Gross *et al*, 2010). The results analyzing the effect of ERK1/2 and PI3K/mTOR inhibitors on dysregulated protein synthesis in FXS were contradictory in these studies, and future, independent studies are needed for validation and clarification. Of interest for their evaluation as therapeutic treatment, a PI3K inhibitor, but not an ERK1/2 inhibitor, could restore mGlu_{1/5}-induced increase in synaptic protein synthesis (Gross *et al*, 2010). Likewise, antagonizing PI3K activity restored mGlu_{1/5}-mediated mTOR activation (Sharma *et al*, 2010). Responsiveness of neurons to external stimuli is an important prerequisite for synaptic plasticity, and a successful therapeutic strategy for FXS will have to not only reduce excess basal signaling, but also restore neuronal response to activity-inducing stimuli. These initial results are therefore promising to further explore PI3K inhibitors as therapeutic targets.

A safety concern for inhibition of PI3K and ERK1/2 in humans is their essential role for cell proliferation and apoptosis. The broad-spectrum PI3K or ERK1/2 antagonists, as used in the discussed studies, could potentially induce severe side effects. Mutations causing aberrant signaling through PI3K/mTOR and/or ERK1/2 have been shown to lead to several different forms of cancer. However, so far, unchanged (Sund *et al*, 2009) or rather decreased (Schultz-Pedersen *et al*, 2001) cancer rates have been reported in patients with FXS. This suggests that in FXS, a very specific function of ERK1/2 and/or PI3K/mTOR signaling is dysregulated, which might be different from cell proliferation and apoptosis. The use of specific antagonists targeting these functions could limit or eliminate

side effects and might, thus, be suitable for pharmacologic treatment in human patients with FXS. In the case of PI3K signaling, promising candidates for specific inhibition are the two FMRP target mRNAs: PIKE and p110 β . Interestingly, p110 β -specific antagonists are already being developed and tested for the treatment of certain forms of cancer (Blajecka *et al*, 2011). Future studies are needed to test whether ERK1/2 pathway components might be promising therapeutic targets in FXS as well, and whether genetic or pharmacologic reduction of p110 β , PIKE, or ERK1/2 can rescue FXS-specific phenotypes in animal models.

Although initial studies showing rescue of FXS-associated phenotypes with ERK1/2 and PI3K antagonists are promising, they will have to be confirmed and extended to more specific antagonists before a potential application in clinical trials with human patients. Importantly, future studies will have to show whether PI3K-specific inhibition can rescue behavioral phenotypes in *Fmr1* KO mice. Furthermore, additional mechanistic studies are needed to solve the current controversies about basal and activity-induced ERK1/2- and PI3K-mediated signaling in the absence of FMRP.

Taken together, these genetic and pharmacologic rescue studies antagonizing different signal transduction molecules in FXS animal models have provided important rationale for novel pharmacologic interventions in FXS. Most of these novel therapeutic strategies will have to be further tested in preclinical studies before clinical tests in humans, but they suggest that in the future, they might become alternative or additional disease-targeted treatments for FXS patients.

THERAPEUTIC INTERVENTION IN FXS

Preclinical data with targeted agents acting on the mGlu_{1/5}, GABA, or other neuronal pathways found to be dysregulated in FXS model systems have been very promising. Although there is much less information on the impact of treatment of humans with FXS with these compounds, early-phase clinical trials have been initiated for some of these targeted treatments. This early translational work is described below, in sections corresponding to mechanistic categories of the proposed targeted treatment.

Agents Reducing Overactive Signaling Between Receptors and the Dendritic Translational Machinery

Agents with extracellular targets. Several mGlu₅ negative allosteric modulators (NAMs) are currently being developed for treatment of FXS and are in clinical trials. Fenobam, the first mGlu₅ NAM used in FXS, was administered in a single oral dose to a cohort of 12 adult males and females with FXS (Berry-Kravis *et al*, 2009). An improvement in the general anxiety level of many of the participants was observed after the dose, although given the trial was open label, this could have been a placebo effect. A significant improvement in PPI was also seen, which would be far less likely because of the placebo effect. There were no safety concerns.

Concurrently, a phase II double-blind placebo-controlled, crossover design trial of AFQ056 (Novartis), with treatment of 30 adult males with FXS for 28 days each with AFQ and placebo, was conducted in Europe (Jacquemont *et al*, 2011). The results of this trial suggested improvement in maladaptive behavior on the Aberrant Behavior Checklist-Community Edition (ABC-C), Clinician Global Impression of Improvement (CGI-I) scale, and the Repetitive Behavior Scale in the subgroup of males with full methylation of *FMR1*. This outcome was adequate to support ongoing development of AFQ056 for treatment of FXS, with a larger multinational double-blind placebo-controlled 3-month trial evaluating the effects of multiple doses of AFQ056 started in the Fall of 2010 (clinicaltrials.gov). Phase I trials of STX107 (Seaside Therapeutics) have been completed in healthy individuals and early treatment trials in FXS are expected in 2011 (clinicaltrials.gov). A double-blind placebo-controlled dose-finding phase II trial of RO4917523 (Roche) in adult males and females with FXS is underway and should be completed by mid-2011 (clinicaltrials.gov). No serious safety concerns have yet emerged in any of these studies, although only a small population of individuals with FXS has thus far been exposed to mGlu₅ NAMs. It has been proposed that mGlu₁ (Koekkoek *et al*, 2005) and muscarinic acetylcholine receptor (Volk *et al*, 2007) NAMs might also be beneficial for individuals with FXS, but these have not been studied. Motor and cognitive side effects were observed in rats exposed to mGlu₁ NAMs (Steckler *et al*, 2005; Kolasiewicz *et al*, 2009), raising concerns about the possible toxicity in human trials.

Intracellular agents. Although a number of intracellular treatment targets have been proposed, in most cases safe and available agents acting on these targets are not yet developed for use in humans. One exception is lithium, for which the preclinical findings in the *dfmr* mutant fly and *Fmr1* KO mouse suggested promise of therapeutic benefit. The effects of short-term (2 months) treatment with lithium were systematically explored for a broad range of phenotypes including behavior, cognition, and biophysical measures in a pilot open-label trial in 15 subjects with FXS (Berry-Kravis *et al*, 2008a) in order to test the concept of inhibition of mGlu_{1/5}-activated translational signaling pathways as a treatment strategy for FXS. Significant improvement in behavior was seen with lithium on the total ABC-C score and three of the ABC-C subscales, the Maladaptive Behavior subscore from the Vineland Adaptive Behavior Scale, a parent visual analog scale for target behaviors, the CGI, and in verbal memory on the RBANS List Learning task. In addition, ERK1/2 phosphorylation rates, shown to be reduced in lymphocytes from humans with FXS (Weng *et al*, 2008), were normalized during lithium treatment (Berry-Kravis *et al*, 2008a), suggesting that ERK1/2 activation rates could be a biomarker for measuring changes in signaling during treatment with agents targeted to receptor-activated translational regulatory pathways. The side effects were generally mild to moderate

and included polydipsia, polyuria, and abnormal thyroid measurements in a few subjects. These data suggested that further studies with a placebo-controlled trial would be indicated; however, such studies have not yet been carried out, partly because of concerns about the chronic toxicity of lithium, but also related to hope that less toxic mechanism-based treatments will be available soon.

Inhibitors of Individual Proteins Excessively Produced or Activated in the Absence of FMRP

Minocycline has been used in clinical trials to inhibit MMP-9, which shows increased activity in the *Fmr1* KO mouse (Bilousova *et al*, 2009). An open-label trial of minocycline in 20 participants with FXS aged ≥ 12 years showed behavioral improvements on the ABC-C, VAS, and CGI (Paribello *et al*, 2010). There were no major side effects but two individuals had to stop treatment because of elevated antinuclear antibodies (ANAs), despite lack of signs of drug-induced lupus. These results have been the rationale for an in-progress double-blind placebo-controlled trial of minocycline in children and adolescents with FXS aged ≥ 5 years. The high risk of irreversible yellow/brown discoloration of the permanent teeth in children aged < 12 years exposed to minocycline, coupled with the idea that targeted treatments might work better if applied at younger ages, has made it crucial to understand the true margin of benefit for this treatment. The other side effects that can be seen at any age include GI symptoms such as vomiting and/or diarrhea (Utari *et al*, 2010), drug-induced lupus, and pseudotumor cerebri.

Agents Activating Surface AMPA Receptors and Activity

A double-blind placebo-controlled trial of effects of CX516 (Cortex Pharmaceuticals) on safety and cognitive and behavioral efficacy measures was carried out in a cohort of 49 individuals with FXS (Berry-Kravis *et al*, 2006). CX516 is a direct AMPA receptor positive modulator or 'ampakine' known to increase LTP and raise BDNF levels, thus potentially increasing surface expression of AMPA receptors (Jourdi *et al*, 2009). Conceptually, it was thought that CX516 would help compensate or correct the AMPA receptor deficit resulting from mGlu pathway overactivity. Realistically, CX516 is a very weak ampakine and thus no improvements were seen except in the group of patients co-treated with an antipsychotic (known to potentiate ampakine activity). This suggests that a more potent ampakine molecule might be helpful in FXS when these become available.

Agents Acting on Other Receptors/Proteins That Regulate Synaptic Activity

In addition to promising findings in FXS animal models with racemic baclofen treatment and evidence that the GABA system is downregulated in the *Fmr1* KO mice,

anecdotal clinical experience in humans with FXS suggested potential behavioral benefits from racemic baclofen, and led to a clinical trial of R-baclofen in humans with FXS. R-baclofen is the enantiomer of racemic baclofen with more potent GABA_B agonist activity. This double-blind placebo-controlled crossover trial (Seaside Therapeutics) with 4-week periods of placebo and active R-baclofen treatment for each subject ($N=63$) did not meet its primary end point of improvement on the ABC Irritability subscale. However, as a phase II exploratory trial targeted to an underlying molecular mechanism rather than a specific behavior, the behavioral target was actually not known. Thus, defining a primary end point *a priori* was difficult, and in *post hoc* analyses the trial did identify a different area of dysfunction in FXS, which R-baclofen appears to target. There was significant improvement during R-baclofen treatment relative to placebo in global preference for treatment period, CGI, the ABC Social Withdrawal subscale, and Vineland Socialization Scale in the subgroup of participants with FXS and more severe impairment in social behaviors (Wang *et al*, 2010b). There were no significant safety issues, and many subjects are continuing treatment though an extension study to evaluate the long-term benefits and toxicity. The positive effects of R-baclofen on social behavior will require confirmation in a larger study, and further development of R-baclofen is underway with two phase III placebo-controlled clinical trials (clinicaltrials.gov).

Other agents acting at an array of receptors have undergone exploratory study in groups with FXS. A pilot open-label study of NMDA antagonist memantine in six participants with FXS showed modest improvement on a CGI in four out of six patients, but no improvement on behavioral rating scales, and two patients showed treatment-limiting irritability (Erickson *et al*, 2009). An open-label study of a sodium channel blocker and glutamate uptake activator riluzole in five patients with FXS showed overall behavioral improvement in only one subject, although ERK1/2 activation rates normalized and there was a suggestion of improvement in hyperactivity symptoms across the group (Erickson *et al*, 2011b). An anecdotal study of three adults with FXS treated with acamprosate, a drug approved for alcohol withdrawal that is a mixed NMDA agonist/antagonist, GABA_A receptor activator, and possible inhibitor of group I mGluR, reported promising improvement in language and behavior in all patients (Erickson *et al*, 2010), but limiting gastrointestinal side effects in one patient. Although aripiprazole is a treatment directed primarily at behavior rather than molecular mechanism, it could theoretically be targeted to dopamine deficits thought to be present in FXS (Wang *et al*, 2008b), given its dopamine agonist activity at lower doses. Aripiprazole has shown good success when used empirically in FXS clinic populations (Berry-Kravis and Potanos, 2004; Hagerman *et al*, 2009), and has resulted in improvement in the ABC Irritability subscore, other ABC subscores, and other behavioral rating scales in a pilot open-label trial in 15 individuals with FXS (Erickson *et al*, 2011a). Plans are

underway to initiate a double-blind placebo-controlled trial of aripiprazole in FXS.

TRIAL DESIGN AND HURDLES IDENTIFIED IN EARLY CLINICAL TRIALS IN HUMANS WITH FXS

Although many neuronal targets for treating the underlying disorder in FXS have emerged, and early translational work has begun, there are still many uncertainties about how to best demonstrate the treatment effects in clinical trials. FXS serves as a good model to develop such designs for other developmental disorders, particularly because FXS is a single genetic disorder in which affected individuals all have the same basic cellular defect, a mouse model is available, some information on synaptic function of FMRP in brain is known, and aspects of FXS model more common disorders with likely mechanistic overlap, including ASDs, ADHD, and learning disabilities.

Some lessons that have been learned from early clinical trials completed to date in FXS subjects are as follows. It is very difficult to identify good cognitive outcome measures for drug effect that can be performed by FXS subjects crossing the entire range of function because of problems with basal scores and ceilings (Berry-Kravis *et al*, 2006, 2009), and more work to validate appropriate measures is needed. Predicting the outcome that will improve most in a complex disorder during initial trials is essentially guesswork, when the treatment targets the underlying disorder and not a specific behavior. Therefore, it is important to run small exploratory trials initially to obtain information from which to choose the primary outcome in subsequent larger trials, and set inclusion criteria to select for subjects with problems in the area targeted by the drug. The factor structure of existing key rating scales such as the ABC (Maltas *et al*, 2011, personal communication) may not be appropriate for FXS if the measure was developed in a non-FXS cohort. This may prevent detection of improvement in a behavioral area because of dilution of scores with items that are not contributing to that behavior. The placebo effect in FXS trials is large (Berry-Kravis *et al*, 2011), most likely because many families are aware of current research. Parents who fill out the rating scales meant to detect medication effect are hoping for and expecting improvement from targeted treatments. Thus, findings from short open-label trials may not be reproduced in placebo-controlled trials. A placebo lead-in (period of placebo treatment for all subjects before randomization to active drug or placebo) is a strategy for FXS trials to limit the placebo effect and allow the treatment effect to be more evident. Recruitment is very difficult and prolonged when concomitant medications are not allowed (Erickson *et al*, 2011a), and thus reasonable concomitant medications that are not predicted to have a major interaction with the agent being studied, will need to be allowed in FXS trials. In fact, this will make a prediction possible about whether the agent being tested can add incrementally to currently existing

therapy. Finally, it appears likely that subgroups of patients will respond better to different targeted agents, even in a uniform disorder such as FXS. There may be biomarkers or markers of clinical severity that define a treatment response just in a subset of patients, as in the socially impaired group in the R-baclofen study (Wang *et al*, 2010b) and the fully methylated group in the AFQ056 study (Jacquemont *et al*, 2011).

The design and evaluation of outcome measures for trials of targeted treatments in FXS and neurodevelopmental disorders has proven to be a major hurdle. Outcome measures chosen need to test a broad ability range to prevent low or high-functioning individuals from showing ceiling or floor effects, overcome problems with cooperation and variable performance, be shown to be reproducible, and quantify core defects and correlate with quality of life and true functional improvement. Only a subset of outcome measures utilized in recent trials have turned out to fulfill the majority of these criteria (Berry-Kravis *et al*, 2006, 2008a, b, 2009) suggesting better, FXS-specific measures are needed. Only recently have investigators begun to develop templates to test the feasibility, reproducibility, and validity assessment before using the measure in a trial (Berry-Kravis *et al*, 2008b; Hessel *et al*, 2009; Knox and Berry-Kravis, 2009; Farzin *et al*, 2011). Choice of outcome measures must also balance use of accepted behavioral measures, which are generally caregiver rating scales (such as the ABC), with precedent for use in drug registration/FDA approval *versus* use of novel measures (Hessel *et al*, 2009; Knox and Berry-Kravis, 2009; Farzin *et al*, 2011) that are more quantitative and may objectively measure core phenotypes and electrophysiology (such as eye tracking or PPI). These novel quantitative measures advance treatment science, but have no precedent for registration, do not clearly predict a specific functional outcome, and are often expensive and difficult to run at multiple sites.

FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

Based on studies in FXS animal models, three major therapeutic strategies in FXS are emerging (summarized in Figure 1): (1) strategies targeting upstream extracellular neurotransmitter receptors, such as metabotropic glutamate, dopamine, and GABA receptors; (2) strategies targeting the intracellular central signal transduction molecules ERK1/2 or PI3K, which integrate and regulate many different signaling pathways, including those stimulating protein synthesis; and (3) strategies targeting multiple further downstream located signaling molecules or proteins translated from FMRP target mRNAs, shown to be dysregulated in FXS (eg, GSK3 β , MMP-9, or PAK). Furthermore, particularly promising groups of FMRP-regulated molecules, for example, those involved in AMPA receptor endocytosis (Waung and Huber, 2009), or

potassium channels might become candidate therapeutic targets in FXS.

Although some of these strategies are already being tested in clinical trials with patients, future studies in FXS animal models are needed to investigate which (or which combinations) of these therapeutic strategies might be the most beneficial for specific FXS patients, each presenting a slightly different phenotype of the syndrome. Ultimately, the value of these complementary therapeutic strategies will strongly depend on the availability of specific drugs that are safe for use in patients and that restore balance of brain signaling.

Several years ago, the Fragile X Clinical and Research Consortium (FXCRC) was created with the support of CDC funding to help ensure state-of-the-art care delivery to meet the needs of individuals with FXS across the country and facilitate large-scale national research efforts. This organization will also allow FXS Clinics across North America to collaborate in preparation for large multisite clinical trials that will be necessary for FDA approval of targeted treatments and to eventually study combinations of therapies.

It is likely that no one agent will be a successful 'cure' for FXS; rather, patients will display optimal responses to different targeted treatments based on individualized complex interactions between genetic variability and neuronal pathway and synaptic function. Thus, it is likely that different patients will function best with certain pathway targets or certain combinations of treatments. There is hope that ongoing development of new targets will gradually build on previous knowledge to result in progressive improvement in treatments to reverse core deficits in all patients with FXS in the future.

Clearly, there is overlap in molecular and synaptic pathways between FXS and autism (Awadalla *et al*, 2010; Pinto *et al*, 2010, see Box 1). Thus targeted treatments for FXS will likely also target dysregulated synaptic mechanisms in a subgroup of patients with autism who have defects in the same pathways that are abnormal in FXS. Successful treatments in FXS are expected to be extended to cohorts with ASDs and other cognitive disorders. Progress in development of these targeted treatments for FXS may result, for the first time, in the possibility of medical intervention to reverse CNS defects and resultant clinical manifestations of developmental cognitive disorders and intellectual disability. The exciting progress made thus far and promise for the continued development of therapeutic strategies results from an in-depth analysis of underlying disease mechanisms and availability of animal models to test restoration of brain functions.

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DISCLOSURE

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(The annotated references were selected based on their importance for the development of therapeutic strategies for FXS. They describe: (1) the discovery of FXS phenotypes in animal models that proved to be useful to develop and/or evaluate therapeutic strategies, (2) rescue of FXS-associated phenotypes using either genetic or pharmacologic strategies in animal models, or (3) important clinical trials with FXS patients. The authors wish to note that this is not intended to depreciate the scientific value of the many excellent basic science studies that were not annotated.)

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