Targeted treatments for fragile X syndrome

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Abstract Fragile X syndrome (FXS) is the most common identifiable genetic cause of intellectual disability and autistic spectrum disorders (ASD), with up to 50% of males and some females with FXS meeting criteria for ASD. Autistic features are present in a very high percent of individuals with FXS, even those who do not meet full criteria for ASD. Recent major advances have been made in the understanding of the neurobiology and functions of FMRP, the *FMR1* (fragile X mental retardation 1) gene product, which is absent or reduced in FXS, largely based on work in the *fmr1* knockout mouse model. FXS has emerged as a disorder of synaptic plasticity associated with abnormalities of long-term depression and long-term potentiation and immature dendritic spine architecture, related to the dysregulation of dendritic translation typically activated by group I mGluR and other receptors. This work has led to efforts to develop treatments for FXS with neuroactive molecules targeted to the dysregulated translational pathway. These agents have been shown to rescue molecular, spine, and behavioral phenotypes in the FXS mouse model at multiple stages of development. Clinical trials are underway to translate findings in animal models of FXS to humans, raising complex issues about trial design and outcome measures to assess cognitive change that might be associated with treatment. Genes known to be causes of ASD interact with the translational pathway defective in FXS, and it has been hypothesized that there will be substantial overlap in molecular pathways and mechanisms of synaptic dysfunction between FXS and ASD. Therefore, targeted treatments developed for FXS may also target subgroups of ASD, and clinical trials in FXS may serve as a model for the development of clinical trial strategies for ASD and other cognitive disorders.

In the past decade, the study of the neurobiology and synaptic mechanisms in fragile X syndrome has emerged as a molecular doorway to future targeted treatments for autism and related developmental disorders. Fragile X syndrome (FXS) provides an excellent model for the translation of basic molecular neuroscience findings to clinical treatment because FXS is a genetically defined condition in which all affected individuals have a uniform single gene defect as the etiology for their condition, an increasing body of information is available regarding the mechanisms through which the genetic defect in FXS impacts molecular events in neurons and resultant synaptic plasticity, a sufficient number of individuals with FXS can be identified to carry out trials, and aspects of FXS model other more common neurodevelopmental conditions such as autistic spectrum disorders (ASDs), learning disability, and attention deficit/hyperactivity disorder (ADHD). Despite the positive aspects of FXS as a translational model, no real template exists for how to approach the translational process, and there are questions about the length of treatment required to impact the underlying disorder, the possibility of developmental windows beyond which treatment cannot be successful, dosing and trial design issues, and the problem of how to measure both short- and

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long-term outcomes, which are ongoing hurdles that must be addressed. Nonetheless, these questions will have to be addressed for targeted treatment of FXS or any neuro-developmental disorder to be successful, and this paper will review the current state of the translational process for targeted treatment in FXS, including known targets, preclinical work with small molecule therapeutics aimed at those targets, active or completed clinical trials, and issues associated with trial design and outcome measures.

Genetics of fragile X syndrome

FXS is the most common known inherited cause of intellectual disability (ID), learning disability and ASD, with an estimated frequency of about 1/2,500–1/4,000 (Hagerman 2008; Turner et al. 1996). FXS results from a trinucleotide repeat (CGG) expansion mutation of >200 repeats (full mutation) in the promoter of the FMR1 (fragile X mental retardation 1) gene (Verkerk et al. 1991), which leads to methylation and transcriptional silencing of the FMR1 promoter with conse-quent loss or significant reduction of expression of the gene product, FMRP (fragile X mental retardation protein; Devys et al. 1993). FMRP is a multifunctional mRNA binding protein involved in the dendritic transport, localization, and translational regulation of several hundreds of mRNA ligands. Therefore, FMRP is thought to regulate translation at the dendrite in response to neural activation, thereby modulating synaptic plasticity and dendritic morphology (for reviews, see Bagni and Greenough 2005; Grossman et al. 2006; Bassell and Warren 2008).

Smaller FMR1 expansions with 55–200 repeats (normal is <45), termed the premutation, are not associated with FMR1 methylation or loss of FMRP expression, but do result in fragile X-associated tremor/ataxia syndrome (Berry-Kravis et al. 2007) or fragile X-associated primary ovarian insufficien-cy (Sullivan et al. 2005). These conditions occur through a presumed RNA toxicity mechanism due to elevated levels of CGG repeat-containing mRNA which accompany the mild reduction in translation of FMRP in the presence of the repeat expansion (Berry-Kravis et al. 2007). Some premuta-tion carriers have been found to have subtle evidence of features that overlap those seen in FXS, including emotional problems such as anxiety, social deficits, obsessive thinking, and/or depression (Hessl et al. 2005; Cornish et al. 2005). A small subgroup of carriers with a larger premutation have mild cognitive disorders and features of FXS, presumed due to uncompensated reduction in translation, with a resultant deficit in FMRP (Tassone et al. 2000).

Because FMR1 is located on the X chromosome, females with a full mutation are more variably affected and, on average, more mildly affected than males due to the production of FMRP from the normal FMR1 allele on the non-mutated X chromosome. Severity of the cognitive impairment and behavioral phenotypes in females with FXS and the full mutation is inversely related to the activation ratio for the normal FMR1 allele and the level of FMRP (Loesch et al. 2002, 2004). Likewise, in males with a full mutation and mosaicism for an unmethylated allele, the severity of the cognitive disorder is related to the amount of unmethylated DNA and FMRP level (Loesch et al. 2002, 2004; Tassone et al. 1999).

Fragile X phenotype

Males with a completely methylated full mutation commonly display mild to moderate ID (Hagerman et al. 2009). Females with the full mutation typically present with learning disabilities, although approximately 25% have ID (de Vries et al. 1996). Physical features include macroorchidism (present in most adult males), and more variably present are prominent ears, macrocephaly, long face, high arched palate, and loose connective tissue leading to hyperextensible joints, flat feet, and soft skin (Hagerman et al. 2009). Medical problems commonly include frequent ear infections, mitral valve prolapse, and seizures (Hagerman et al. 2009). Males with FXS have characteristic behavioral features including hyperactivity, impulsivity, attention problems, anxiety, 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 (Hagerman et al. 2009; Berry-Kravis and Potanos 2004; Wang et al. 2010a). They 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); abnormal sensory gating can be demonstrated in prepulse inhibition studies (Hessl et al. 2008a). Anxiety disorders are common in both males and females with FXS, including selective mutism, separation anxiety, social phobia, and specific phobias (Hagerman et al. 2009; de Vries et al. 1996; Sullivan et al. 2007), and there is often generalized anxiety with multiple specific areas of difficulty. Aggression occurs in at least 30–50% of males and is most commonly seen in adolescence (Hessl et al. 2008b).

Females with FXS exhibit more variable and less frequent involvement with respect to physical features and medical problems, but often have attention problems, impulsivity, and executive function deficits even when their IQ is in the normal range (Hagerman et al. 2009; de Vries et al. 1996; Berry-Kravis and Potanos 2004; Wang et al. 2010a). Shyness, selective mutism, specific phobias, social anxiety, and social deficits are common in females (Berry-Kravis and Potanos 2004; Wang et al. 2010a).

Molecular neurobiology and synaptic pathology in FXS

FMRP is an mRNA-binding protein involved in the transport, localization, and translational regulation of a subset of dendritic mRNAs (Darnell et al. 2001; Brown et al. 2001; Chen et al. 2003; Miyashiro et al. 2003). Many of the known interactions of FMRP at the synapse are shown in Fig. 1a. The protein has two hnRNP K homology domains and one RGG box as well as nuclear localization and export signals. The RGG box of FMRP mediates interactions with G-quartet sequences in mRNA ligands (Darnell et al. 2001), although there appear to be other mechanisms for FMRP-mRNA interactions as well. FMRP is predominantly found in dendritic spines associated with polysomes (Feng et al. 1997) or non-translating ribonucleoprotein (RNP) particles known as processing bodies (PB; Ceman et al. 1999; Zalfa et al. 2003; Blackwell et al. 2010; Weiler et al. 1997). In the PB, FMRP interacts with the FMRP homologs FXR1 and FXR2 and other proteins including Argonaute and CYFIP1, mRNA decapping enzymes, the guide RNA BC1, miRNAs, and mRNA targets (Bagni and Greenough 2005; Zalfa et al. 2003); in concert with these other proteins and RNAs, FMRP mediates a predominantly repressive effect on the translation of its mRNA cargos. Over 500 mRNA cargos of FMRP have been identified (Darnell et al. 2001; Brown et al. 2001; Chen et al. 2003; Miyashiro et al. 2003), with many coding for proteins thought to influence synapse formation and synaptic plasticity.

After transport to dendrites and spines, FMRP binds to mRNAs (including its own) and regulates their translation in response to neural activation (Weiler et al. 1997, 2004). Specifically, FMRP appears to regulate translation pathways activated by group 1 metabotropic glutamate receptors (mGluR1 and mGluR5; Huber et al. 2002; Antar et al. 2004; Aschrafi et al. 2005) and muscarinic (M1) acetylcholine receptors (Volk et al. 2007) and possibly has a more general function in the regulation of translational activation by multiple synaptic Gq-linked receptors, including dopamine D1 receptors (Wang et al. 2010b). Activation of these receptors transiently induces FMRP dephosphorylation (Narayanan et al. 2008), which results in the reduction of FMRP interaction with Argonaut (AGO2) and microRNAs in the RISC complex and increased interaction with Dicer (Cheever and Ceman 2009) and, hence, loss of interaction with bound mRNAs, thereby relieving the repressive effect of FMRP on the translation of these mRNA targets, leading to the production of a pulse of new protein (Qin et al. 2005). Some of the FMRP-regulated proteins, in particular STEP (Zhang et al. 2008) and Arc (Park et al. 2008), are likely responsible for AMPA receptor endocytosis and resultant maintenance of translation-dependent group 1 mGluR-induced long-term depression (LTD) in hippocampus, as well as other receptor-activated translation-dependent forms of LTD and long-term potentiation (LTP) throughout the brain.

In the fmr1 knockout mouse, FMRP is absent, and there is both constitutively elevated translation of FMRP target mRNAs (~2-fold normal) and a loss of the translation "pulse" after mGluR stimulation (both due to loss of baseline translational repression when FMRP is not present). Thus, levels of synaptic proteins corresponding to a number of FMRP target mRNAs have been shown to be constitutively elevated in the fmr1 knockout mouse, including MAP1B, PSD95, CaMKII, STEP, PIKE, amyloid precursor protein (APP), Arc, PP2A, potassium channel Kv3.1b, and others (Darnell et al. 2001; Brown et al. 2001; Chen et al. 2003; Miyashiro et al. 2003; Park et al. 2008; Zhang et al. 2001; Muddashetty et al. 2007; Goebel-Goody et al. 2010; Westmark and Malter 2007; Shumbos et al. 2010). The levels of many of these proteins have also been shown to be unresponsive to group I mGluR activation. This dysregulated dendritic expression (either temporally, spatially, or quantitatively) of multiple FMRP mRNA ligands results in abnormal synaptic plasticity, including enhanced mGluR-activated hippocampal (Huber et al. 2002) and cerebellar (Koekkoek et al. 2005) LTD and impaired LTP in the hippocampus (Lauterborn et al. 2007), cortex (Li et al. 2002; Larson et al. 2005), and amygdala (Subrathan et al. 2010). Other expected consequences of excessive constitutive activation of mGluR-mediated dendritic protein synthesis are also found in the fmr1 knockout (KO) mouse, including reduction of synaptic AMPA receptors (Koekkoek et al. 2005; Lauterborn et al. 2007), abnormal epileptiform discharges (Chuang et al. 2005), and abnormal dendritic spine morphology (Grossman et al. 2006; Comery et al. 1997; Irwin et al. 2002; Nimchinsky et al. 2001).

Indeed, the cerebral cortex of adult fmr1 KO mice (Grossman et al. 2006; Chuang et al. 2005; Comery et al. 1997; Irwin et al. 2002; Nimchinsky et al. 2001) and autopsy specimens from individuals with FXS (Irwin et al. 2001) both show increased density of long, thin, tortuous postsynaptic dendritic spines which are considered "immature" and are normally seen in early neocortical development as well as reduction of mature, short mushroom-shaped spines. This pattern appears to represent a deficit in the "pruning" of unnecessary synaptic contacts, suggesting that FMRP is required for the important processes of synapse stabilization and pruning during synapse maturation. The morphological abnormalities and synaptic plasticity deficits found in the fmr1 knockout mouse are associated with numerous cognitive, behavioral, and electrophysiological phenotypes, including abnormal ocular dominance plasticity (Dolen et al. 2007), olfactory learning deficits (Larson et al. 2008), impaired memory formation

(Ventura et al. 2004; Brennan et al. 2006), decreased motor learning (Koekkoek et al. 2005), increased open-field hyperactivity (Yan et al. 2004) and abnormal marble burying (Paylor 2008), abnormal social behaviors (Paylor 2008; Mines et al. 2010; Spencer et al. 2008), abnormal prepulse inhibition (PPI; Paylor et al. 2008; de Vrij et al. 2008), prolonged epileptiform bursts (Subrathan et al. 2010), neuronal network hyperexcitability (Gibson et al. 2008), audiogenic seizures (Yan et al. 2005), abnormal growth patterns (Dolen et al. 2007), and increased protein synthesis (Qin et al. 2005).

Findings in the *Drosophila* model of FXS, in which there is loss of *dfmr1* (homolog of the *FMR1* gene in the *Drosophila* genome) activity (Zhang et al. 2001) parallel those in the knockout mouse and include defects in circadian rhythms, synaptic branching, courtship behavior, and learning (Zhang et al. 2001; McBride et al. 2005; Dockendorff et al. 2002; Morales et al. 2002). Furthermore, many phenotypic features of FXS in humans resemble or overlap those seen in the mouse and fly models, including seizures, electrical excitability on EEGs, hypersensitivity to tactile stimuli, cognitive difficulty, strabismus, enhanced anxiety, coordination problems and loose stools, and are effects that have been proposed to occur in a setting of exaggeration of mGluR-mediated protein synthesis-dependent processes that would normally be inhibited by FMRP (Bear et al. 2004; Bear 2005).

The pathways through which group 1 mGluRs and other forms of neural stimulation activate translation show abnormal activity in the *fmr1* knockout, suggesting the activity levels in these pathways themselves, are under the control of FMRP. Group 1 mGluRs regulate dendritic translation through several pathways (Waung and Huber 2009). One pathway involves the activation of phospholipase C (PL-C), with resultant production of IP3 and DAG from PIP2, leading to the activation of PKC and induction of the mitogen-activated protein kinase kinase pathway through the phosphorylation of MEK, extracellular signal-regulated kinase (ERK), and Mnk1 in sequence, leading to translational activation through eIF4E. The second pathway involves signaling through homer-linked PIKE/PI3K, with conversion of PIP2 to PIP3, which then recruits pleckstrin homology-containing kinases PDK1 and Akt to the membrane where they are phosphorylated, with subsequent activation of mTOR and phosphorylation of 4EBP leading to the release of eIF4E to form eukaryotic initiation factor complex 4F and activation of CAP-dependent translation. mTOR activation also results in the phosphorylation of p70S6 kinase, which activates TOP-dependent translation. The PI3K enhancer, PIKE, is a predicted mRNA target for FMRP and in fact is found to be elevated in the *fmr1* knockout, with resultant downstream activation of Akt, mTOR, S6 kinase, and 4EBP (Sharma et al. 2010). A third

pathway involves direct Homer interaction and phosphorylation of EF2 kinase, which inhibits translational elongation and affects translational regulation (Waung and Huber 2009). Furthermore, excessive dephosphorylation of GSK3ß to the inactive form of the enzyme (presumably due to higher levels of phosphatase PP2A, in the *fmr1* knockout) occurs in *fmr1* knockout mouse brain, is dependent on elevated signaling through group 1 mGluRs, and may mediate and/or result from disturbances of translational regulation in the absence of FMRP (Min et al. 2009). Thus, in the *fmr1* knockout, translational activation due to the absence of direct FMRP-mediated ribosomal inhibition and stalling is compounded by an increase in the activity of translation-activating cascades because of increased basal levels of synthesis of FMRP-target proteins that participate in those cascades.

Additional complex changes in molecular events at the synapse occur in the absence of FMRP, in many cases because of increased basal activity of proteins representing FMRP target proteins, lack of appropriate sensitivity to synaptic activation, direct protein–protein interactions of FMRP, or other yet unknown mechanisms. Examples of identified synaptic disturbances include FMRP interaction with dRac, cAMP, and the potassium channel Slack. The small GTPase dRac is a ligand of the fly dFMR protein and activates p21-activated kinases (PAKs). It has been proposed that excessive translation of Rac in the absence of repression by FMRP might lead to the upregulation of PAK, with resultant excessive phosphorylation of ERK (also observed in the fmr1 knockout), and this might mediate some of the cellular effects of FMRP deficiency (Lee et al. 2003; Hayashi et al. 2007). PAK associated with PSD95 at the synaptic density (although not total PAK) has been shown to be increased in the *fmr1* knockout, but PAK fails to activate with theta burst afferent stimulation (TBS) as it does in wild-type mice, leading to defects in actin polymer stabilization, spine morphology, and TBS-induced hippocampal LTP (Chen et al. 2010). Cells from the *fmr1* knockout mouse and from individuals with fragile X show reduced cAMP production

Fig. 1 Synaptic translation and signaling pathways modulated by FMRP (**a**) and dysregulation of these pathways in the absence or significant reduction of FMRP (**b**). *Shaded areas* in (**b**) indicate groups of targets for different strategies aimed at treatment of FXS by correcting dysregulated neuronal pathways. *Shaded areas* are numbered according to type of treatment strategy and correspond to numbering system for treatment strategies in the text as follows: (*1*) reduction of activity in pathways that transduce signals from group 1 mGluRs or other Gq-linked receptors to the dendritic translational machinery via (*1a*) extracellular pathway (receptor) blockers and/or (*1b*) intracellular pathway blockers; (*2*) reduction of activity of individual proteins regulated by FMRP; (*3*) increasing surface AMPA receptors and/or activity; (*4*) modification of activity of other receptors/proteins that regulate synaptic activity; and (*5*) blocking translation of mRNAs regulated by FMRP using antisense technology
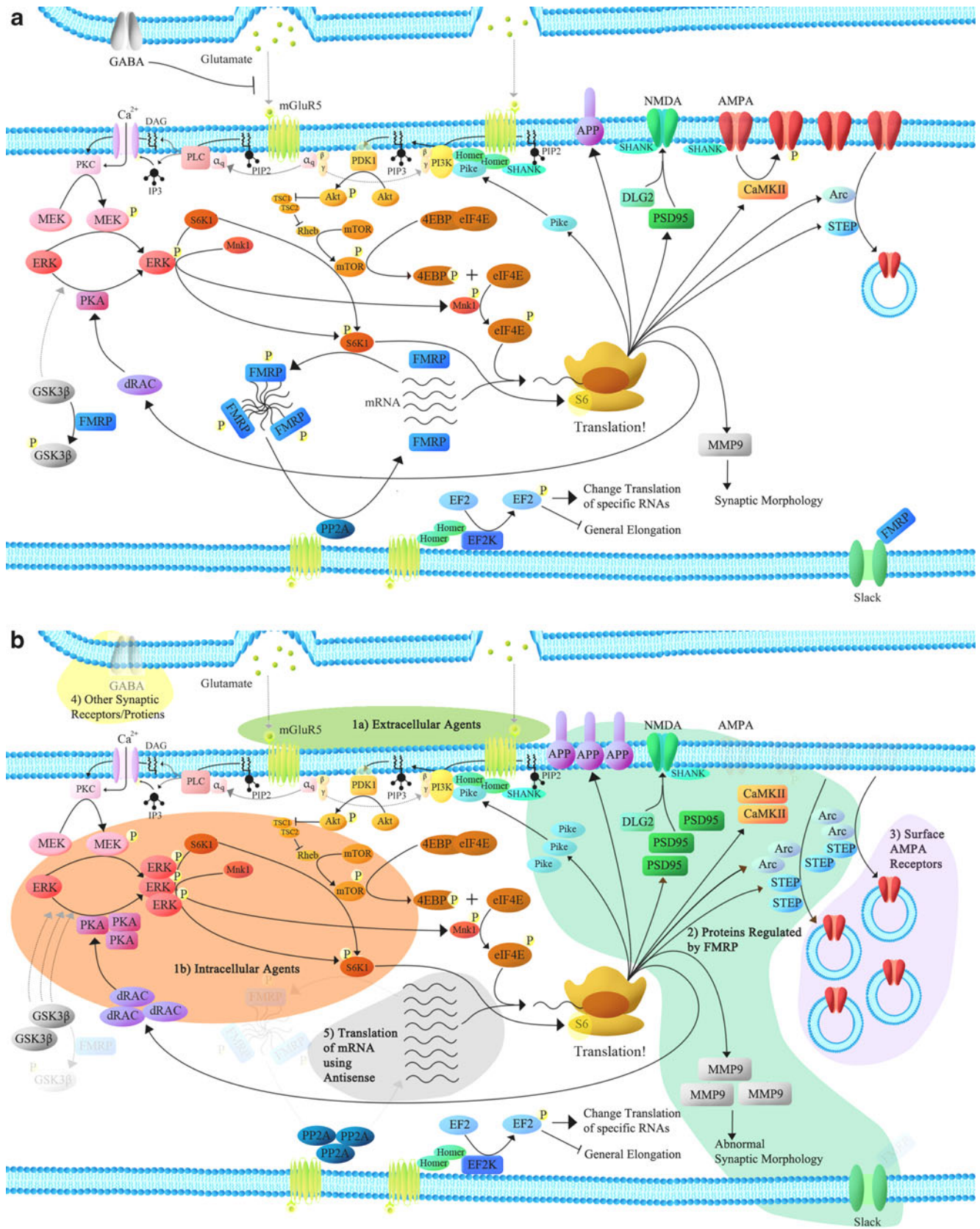
(Chen et al. 2010; Berry-Kravis and Hicar 1995; Kelley et al. 2007) which is dependent on FMRP levels (Berry-Kravis and Ciurlionis 1998). Likewise, adenylate cyclase activity modulates mGluR-mediated regulation of FMRP activity (Wang et al. 2008a). Although the mechanism through which FMRP regulates cAMP production is not known, FMRP is known to bind adenylate cyclase subunit mRNAs (Darnell et al. 2010). FMRP has recently been shown, for the first time, to directly interact with a membrane protein by binding the C-terminus of the sodium-activated potassium channel Slack and activating the channel (Brown et al. 2010). This suggests that FMRP has synaptic activities that are distinct from the role in translational regulation and thus may be very diverse.

Numerous receptor system alterations have been reported in the *fmr1* knockout, including the following examples. Synaptic GABA system proteins show decreased levels of GAD and GABA-A receptor subunits α1,3 and 4, ß1 and 2, and δ1 and 2 in several studies (D'Hulst et al. 2006; Gantois et al. 2006; D'Hulst and Kooy 2007). In another report, GABA-A α1, ß2 and δ, and GAD were downregulated at different specific times during development in the knockout (Adusei et al. 2010), overall suggesting the potential for reduced inhibitory signaling and inhibitory/excitatory imbalance in FXS. There is additional evidence for dopamine D1 receptor signaling deficits in the *fmr1* knockout brain (Wang et al. 2008b). New evidence has recently emerged demonstrating that group I mGluR-dependent protein synthesis-independent endocannabinoid (eCB) LTD is abnormal in the *fmr1* knockout mouse, likely due to abnormal regulation of production and levels of endocannabinoid 2-AG at synapses (Zhang and Alger 2010; Maccarone et al. 2010). Since eCB-producing neurons are widely distributed in the brain, this may be an important mechanism impacting synaptic function in FXS

Targets, therapeutics, and preclinical work

The model of cognitive and behavioral manifestations in FXS as the result of downstream effects of excessive translation normally activated by mGluR or other Gq-coupled receptor activation, in the absence of FMRP, has led to the identification of numerous possible treatment targets, listed in Table 1 and depicted in Fig. 1b (Wang et al. 2010a; Bear et al. 2008; Levenga et al. 2010; Heulens and Kooy 2011), directed at (1) reducing activity in pathways that transduce signals from group 1 mGluRs or other Gq-linked receptors to the dendritic translational machinery, (2) reducing the activity of individual proteins regulated by FMRP, (3) increasing surface AMPA receptors and activity, (4) modifying the activity of other receptors/proteins that regulate synaptic activity, and (5) blocking translation of mRNAs regulated by FMRP using antisense

technology. Success of treatments aimed at these targets has been tested in cell culture and in animal models of FMRP deficiency, including both the *dfmr* mutant fly and *Fmr1* knockout mouse models. Indeed, these models have been proven to be powerful systems to elucidate the causes of FXS and identify effective therapeutics (Bakker and Oostra 2003). Targeted treatments have shown success in model organisms even in adulthood (Yan et al. 2005; McBride et al. 2005), consistent with the finding that induction of expression of FMRP in adult *fmr1* conditional knockout mice leads to the reversal of most phenotypes (Nelson 2010), suggesting that there may not be an absolute developmental requirement for FMRP and that deficits due to the absence of FMRP can be reversed with intervention to correct the synaptic dysfunction when applied in adulthood. Preclinical testing of targeted treatments in animal models is presented below, in subsections defined by the mechanism of the targeted treatment.

Signaling from receptor to dendritic translational machinery

Treatment targets in this category can be divided into those that act extracellularly on receptors to decrease translational signaling and those that act intracellularly on the signaling cascade. Extracellular agents generally target a particular receptor and thus are more specific treatments whose effects are often limited to brain or even just specific subsets of neurons. Intracellular agents have the advantage of potentially normalizing signaling resulting from the activation of the pathway through any receptor, and thus may target the mechanism more generally than receptor-specific molecules. On the other hand, intracellular agents predominantly target proteins with substantial overlap in multiple cellular signaling pathways, including those for growth and specification and other hormonal responses; therefore, these agents will be prone to an increased likelihood of off-target effects as well as cellular and organ toxicity.

Extracellular agents

Potential extracellular agents considered thus far include group 1 mGluR receptor (particularly mGluR5) blockers and muscarinic (M1) acetyl choline receptor blockers. Of the group 1 mGluRs, mGluR1 receptors are present mainly in the cerebellum and hippocampus, while mGluR5 receptors are present throughout the brain except in the cerebellum (Bear et al. 2004; Bear 2005). The broader distribution of mGluR5 receptors, in conjunction with toxicity in the form of motor deficits observed in animal models treated with mGluR1 blockers, has pointed to mGluR5 receptors as the better initial target for the pharmacotherapy of FXS.

In cultured neural cells, knockdown of FMRP using antisense oligonucleotides directed at FMR1 mRNA results

Table 1 FXS targeted treatments in models and man

Agent/target	Phenotypes reversed		Translational progress
	dfmr mutant fly	*Fmr1* KO mouse	Humans with FXS
(1)a Block translational signaling pathway—external			
mGluR5 inhibition (MPEP, fenobam, STX107, AFQ056, RO4917523); MPEP used in models except where marked[a]	Courtship behavior—immediate recall and short-term memory; mushroom body formation; odor-shock memory; survival on glutamate-containing food	Audiogenic seizures[a]; Epileptiform bursts; open-field hyperactivity; dendritic spine morphology[a]; amygdala mEPSP frequency; prepulse inhibition[a]; marble burying[a]	Fenobam—phase IIa single-dose open-label trial—PPI improved, anxiety reduced; AFQ056—phase II[b] trial completed with improvement in fully methylated patients, phase III trial being initiated; RO4917523—phase II trial in progress STX107—phase I completed
mGluR5 inhibition by genetic reduction of mGluR5 receptors		Audiogenic seizures; dendritic spine density; excessive protein synthesis; abnormal growth pattern; ocular dominance plasticity; inhibitory avoidance extinction	
(1)b Block translational signaling pathway- internal			
Lithium (inhibition of GSK3β and PI turnover)	Courtship behavior—immediate recall and short-term memory; mushroom body formation	Audiogenic seizures; open-field hyperactivity; dendritic spine morphology; learning and anxiety deficits in the elevated plus maze, elevated zero maze, passive avoidance; social interaction deficit with new mice and anxiety-related behaviors during social interaction	Open label trial—behavioral improvement, some adaptive skills and verbal memory improved; ERK biomarker normalized
GSK3β inhibition (AR-A014418 or SB-216763)		Audiogenic seizures	
PAK inhibition by genetic reduction of PAK		Dendritic spine morphology; Cortical LTP deficits; open-field hyperactivity, repetitive behaviors, center field anxiety deficit; Fear conditioning	
PI3K inhibition (LY294002)		Dendritic spine morphology; mTOR overactivity	
ERK/MEK inhibition (SL327)		Audiogenic seizures; protein synthesis	
(2) Inhibit activity of individual FMRP-regulated proteins			
Inhibit MMP9 (minocycline)		Dendritic spine morphology; anxiety in elevated plus maze; exploratory behavior in Y maze	Improvement in behavior in small open-label trial
Inhibit APP/Aβ with antibody or by genetic reduction of APP		Audiogenic seizures; dendritic spine morphology; marble burying	
Inhibit STEP by genetic reduction of STEP		AMPA receptor internalization; audiogenic seizures; open-field hyperactivity	
(3) Activate surface AMPA receptors			
Ampakines (CX516, CX614)		CX614 increases BDNF which reverses impairments in hippocampal TBS-LTP	CX516—phase II trial—no cognitive or behavioral effects overall—dose too low but may have helped subjects co-treated with antipsychotics

Table 1 (continued)

Agent/target	Phenotypes reversed		Translational progress
	dfmr mutant fly	*Fmr1* KO mouse	Humans with FXS
(4) Other synaptic receptors/proteins			
GABA-B agonists (baclofen, R-baclofen)	Survival on glutamate-containing food; memory deficits	Audiogenic seizures; open-field hyperactivity; marble burying	R-baclofen phase II trial—improvement in overall function, social and language function in more socially impaired subject group
GABA-A agonists (ganaxolone)		Audiogenic seizures	
Anticholinesterase (donazepil)			Open label trial -behavioral and social improvement
NMDA antagonists (memantine, acamprosate)			Memantine—small open-label trial—no overall improvement
			Acamprosate—open-label trial in 3 patients with improved language and socialization
Glutamate uptake inhibition (riluzole)			Riluzole—small open-label trial—no overall improvement, ERK biomarker normalized

[a] Audiogenic seizures: MPEP, fenobam, and STX107; spine shape: MPEP, fenobam, AFQ056; PPI: MPEP, fenobam, AFQ056; motor learning: MPEP, fenobam, AFQ056; marble burying: MPEP, fenobam, STX107

[b] All phase II or III trials listed in the table are placebo-controlled double-blind trials, unless otherwise noted

in increased AMPA receptor (GluR1) internalization, presumably due to mGluR5 pathway overactivity as 2-methyl-6-(phenylethynyl)-pyridine (MPEP), an mGluR5 blocker, is able to reduce AMPA internalization back to normal levels (Nakamoto et al. 2007). This finding suggests that synaptic cellular deficits due to the absence of FMRP can be reversed by the inhibition of mGluR5-mediated activity. In the *dfmr* mutant fly, treatment with MPEP has been shown to reverse impairments in naïve courtship behavior, immediate recall and short-term memory (McBride et al. 2005), mushroom body formation (McBride et al. 2005), odor-shock memory (Bolduc et al. 2008), and survival on glutamate-containing food (Chang et al. 2008). MPEP and other mGluR5 negative modulators (including fenobam, AFQ056, and STX107) have been shown to reverse multiple phenotypes including audiogenic seizures (Paylor 2008; de Vrij et al. 2008), epileptiform bursts (Chuang et al. 2005), open-field hyperactivity (de Vrij et al. 2008), dendritic spine shape (Spencer et al. 2008), prepulse inhibition (Spencer et al. 2008), and behavioral phenotypes including abnormal marble burying (Paylor 2008). Treatment with MPEP, however, only partially corrects synaptic plasticity deficits in the *fmr1* knockout amygdala, with failure to rescue LTP deficits and excessive AMPA receptor internalization, but successful rescue of mEPSC frequency (Subrathan et al. 2010).

Additional support for the mGluR5 receptor as a treatment target in FXS has come from experiments in which *fmr1* knockout mice also heterozygous for a null mutation in the gene coding for the mGluR5 receptor were generated, resulting in a 50% reduction in mGluR5 expression, in addition to loss of FMRP in these animals (Dolen et al. 2007). Numerous phenotypes were rescued by genetically reducing mGluR5 expression in these *fmr1* KO mice, including abnormal ocular dominance plasticity, increased density of dendritic spines on cortical pyramidal neurons, increased basal protein synthesis in the hippocampus, exaggerated inhibitory avoidance extinction, audiogenic seizures, and accelerated body growth (Dolen et al. 2007). The accumulated data showing both pharmacological and genetic reversal of *fmr1* knockout phenotypes have provided substantial support for the proposal of Bear et al. (Bear et al. 2004, 2008; Bear 2005) that the excessive mGluR5 signaling in the *fmr1* knockout model could be at least partially responsible for many of the psychiatric and neurological symptoms, such as poor cognitive development, seizures, anxiety, movement disorders (stereotypic motor movements), and even accelerated body growth seen in FXS.

It has been proposed that mGluR1 negative modulators might also be beneficial for development in FXS, but little preclinical information is available regarding the effects of these molecules in the *fmr1* knockout. These molecules are associated with substantial negative cognitive and motor effects in normal rats (Kolasiewicz et al. 2009; Steckler et al. 2005) and hence are not in clinical development;

however, they remain possible targeted treatments in FXS due to the specific disease defect of excessive signaling through mGluR1 receptors.

Intracellular agents

These agents target the pathway for derepression of translation after mGluR activation and include lithium, PI3K inhibitors, GSK3β inhibitors, and PAK inhibitors, among others. Lithium may attenuate activation of the PL-C signaling pathway by inhibiting phosphatidyl inositol (PI) turnover (Berridge 1993) and clearly inhibits GSK3β activity (Min et al. 2009; Yuskaitis et al. 2010), which would decrease phosphorylation of ERK and multiple signaling molecules that regulate translation; all of these effects would theoretically lead to the reduction of translational activation. Lithium has been shown to improve defects in naïve courtship behavior, immediate recall, and short-term memory in *dfxr* mutant flies (McBride et al. 2005) and to reverse phenotypes including audiogenic seizures (Min et al. 2009), open-field hyperactivity (Min et al. 2009; Liu et al. 2010), deficits on a social interaction task (Mines et al. 2010; Liu et al. 2010), learning deficits (Yuskaitis et al. 2010; Liu et al. 2010), anxiety (Yuskaitis et al. 2010; Liu et al. 2010), novel object recognition (Venkitaramani et al. 2010), and dendritic spine shape (Liu et al. 2010) in the *fmr1* knockout mouse model. Given that lithium treatment normalizes levels of activated ERK and GSK3β in the *fmr1* knockout (Venkitaramani et al. 2010), it appears that the main effect of lithium in the *fmr1* knockout is to reduce excessive GSK3β activity with resultant reduction in excessive ERK-mediated translation; however, lithium may also directly increase surface expression of AMPA receptors (Du et al. 2010) and reduce excess MAP1B activity (Owen and Gordon-Weeks 2003). Other GSK3β inhibitors such as SB-216763 can reverse these phenotypes as well (Min et al. 2009). Effects of GSK3β inhibitors are not additive with those of mGluR5 blockers, suggesting that excess GSK3β activity is due to excessive activity in mGluR5-activated pathways (Min et al. 2009).

It was noted that PAK-deficient mice had dendritic spine pathology opposite that observed in the fmr1 knockout, with excessive short stubby spines and reduced density of spines. Hence, in double-mutant *fmr1* knockout/PAK-deficient mice (expressing a dominant negative PAK transgene in the forebrain), rescue of *fmr1* knockout phenotypes, including spine morphology abnormalities, cortical LTP deficits, and behavioral abnormalities, was observed (Hayashi et al. 2007). It was proposed that because PAK plays a role in ERK activation, PAK deficiency may correct excessive ERK activation and signaling and thus partially correct translational activation and resultant phenotypes in the *fmr1* knockout. This is consistent with the finding that reduction

of ERK activation with SL-327 normalizes protein synthesis and blocks audiogenic seizures in the *fmr1* knockout mouse (Osterweil et al. 2010), and small molecules that inhibit ERK have been proposed as possible targeted therapeutics in FXS on the basis of these findings.

Elevated PI3K activity observed in the *fmr1* knockout can be reduced using PI3K antagonist LY294002, resulting in the reversal of dendritic spine phenotypes (Gross et al. 2010), normalization of aberrant mTOR activation, and restoration of sensitivity of mTOR to mGluR activation with 3,4-dihydroxyphenylglycine (DHPG; Sharma et al. 2010). Thus, agents acting in this pathway including PI3K, mTOR, and S6 kinase inhibitors may also be potential treatment targets.

Taken together, the above findings suggest that deficits in receptor-activated signaling pathways in the absence of FMRP are beginning to be sufficiently well understood as to generate many potential targets for eventual treatment of fragile X syndrome.

Activity of individual proteins regulated by FMRP

Treatment targets in this category would be key synaptic proteins (other than those in the translational signaling pathway discussed above) for most of which the mRNA is an FMRP cargo, and the protein has been shown to be excessively translated in the absence of FMRP, with resultant elevated basal levels of protein, typically also with loss of responsiveness of protein translation/levels to mGluR activation with DHPG. Even though only one misregulated protein would be targeted, the protein would have sufficient key synaptic activity that correction of levels would be at least partially helpful in reversing phenotypes. Examples of targets in this category that have been studied include matrix metalloprotein-9 (MMP9; Bilousova et al. 2009), APP (Westmark and Malter 2007), and STEP kinase (Goebel-Goody et al. 2010). Excessive activity of MMP9 was demonstrated in the hippocampus of the *fmr1* knockout mouse relative to wild type, and minocycline, an antibiotic that inhibits MMP9, currently used predominantly for acne in teenagers, was found to normalize dendritic spine phenotypes in both in vivo and in cultured hippocampal neurons from the *fmr1* knockout as well as to improve anxiety in the elevated plus maze and exploratory behavior in the Y maze (Bilousova et al. 2009). APP and metabolites have been shown to be elevated in the *fmr1* knockout, and either pharmacological (with antibodies to APP/Aβ) or genetic (creation of heterozygous APP null, *fmr1* knockout double mutant) reduction of APP results in at least partial reversal of audiogenic seizure, behavioral, and spine phenotypes (Westmark et al. 2010). STEP levels are increased in the fmr1 knockout; in fact, STEP may be a key LTD protein produced by translational activation, and which then is responsible for the internalization of AMPA

receptors. STEP/*fmr1* double knockout animals show rescue of open-field hyperactivity and audiogenic seizure phenotypes seen in the *fmr1* knockout, and STEP inhibitors have been proposed as a potential targeted treatment (Park et al. 2008).

An example of a different type of target would be a protein regulated by FMRP directly rather than through control of translation of its mRNA. One such example would be Slack, a sodium-activated potassium channel subunit that is activated by FMRP through direct interaction of FMRP with the C-terminal of Slack (Brown et al. 2010). In the *fmr1* knockout, current through this channel is significantly reduced relative to wild type, suggesting that activators of Slack-containing channels might be a potential therapeutic strategy (Brown et al. 2010), although no studies of the therapeutic effects of such agents have yet been conducted.

Surface AMPA receptors and activity

Agents in this category are AMPA activators or "ampa-kines" and could be utilized to increase deficient synaptic AMPA receptor activity by direct positive modulation of receptor activity in the presence of glutamate and/or inducing BDNF to increase the number of surface AMPA receptors, thus normalizing deficient LTP. The so-called ampakine CX614 has been shown to increase BDNF in the hippocampus of the *fmr1* knockout and wild-type mouse. BDNF, itself, reverses hippocampal TBS-induced LTP deficits in the *fmr1* knockout (Lauterborn et al. 2007). Behavioral effects of these treatments have not yet been measured in the *fmr1* knockout.

Other receptors/proteins that regulate synaptic activity

The main group of agents in this category that have been thus far studied in FXS animal models are GABAergic agents aimed at correcting the reduced activity of GABA inhibitory systems and reestablishing a balance between inhibition and excitation in the brain. GABA-A agonists would be directed at compensating for GABA-A subunit deficiencies (D'Hulst et al. 2006; Gantois et al. 2006), while GABA-B agonists act presynaptically to block glutamate release and thus would tend to decrease group I mGluR activation and excessive signaling, although it is likely that GABA-B agonists have a complex mechanism involving postsynaptic effects as well (Sohn et al. 2007). In the *dfmr* mutant fly, a variety of GABA agonists rescue the lethality phenotype from glutamate-containing food, as well as memory deficits and neuropathological phenotypes (Chang et al. 2008). In the *fmr1* knockout mouse, racemic baclofen, a GABA-B agonist, rescues the audiogenic seizure phenotype (Pacey et al. 2009). The R-baclofen enantiomer of baclofen appears to have substantially better potency than S-baclofen, and R-baclofen reverses several behavioral phenotypes, including marble burying and open-field hyperactivity as well as audiogenic seizures (Paylor 2008). The GABA-A agonist ganaxolone (Carter et al. 1997) reduces audiogenic seizures in the *fmr1* knockout (Kooy 2010), but no other effects have yet been explored.

Additional receptor targets would theoretically include mGluR2/3 agonists or antagonists, through mechanisms having to do with the regulation of synaptic glutamate levels, and blockers of endocannabinoid degradation, which would increase defective mGluR-dependent endocannabinoid-mediated LTD observed in the *fmr1* knockout (Zhang and Alger 2010; Maccarrone et al. 2010). Preclinical testing of these molecules in the knockout has not yet been accomplished.

Translation of mRNAs regulated by FMRP using antisense technology

This strategy for developing treatment targets was recently proposed when it was discovered that mir125a, a small RNA involved in translational silencing of specific messages, operates in conjunction with phosphorylated FMRP and AGO2 to bind to PSD-95 mRNA and block translation of PSD-95. DHPG and anti-mir125a increase the translation of PSD-95 by dissociating mir125a from PSD-95 mRNA. In the absence of FMRP, mir125a is reduced in inhibitory complexes in synaptoneurosomes and PSD-95 translation is increased and uncoupled from mGluR activation (Mudda-shetty et al. 2010). This has suggested that the delivery of specific siRNAs like mir125a that target FMRP cargos to *fmr1* knockout dendrites might be a new therapeutic strategy that could compensate for the absence of FMRP by reducing (normalizing) the translation of their target messages (Muddashetty and Bassell 2009).

Clinical trial experience with targeted treatments in humans with FXS

Taken together, the preclinical data with agents acting in the above mechanistic categories of targeted treatment 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 and even completed for treatments in many of the categories (listed in Table 1).

Signaling from receptor to dendritic translational machinery

Extracellular agents

Several mGluR5 negative modulators are currently being developed for the treatment of FXS and are in clinical trials,

but are not available for general use in humans. Fenobam, the first mGluR blocker used in FXS, was administered in a single oral dose to 12 adult males and females with FXS (Berry-Kravis et al. 2009). In this trial, an improvement in the general anxiety level of many of the participants was observed after the dose, although given that 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 to be due to 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). Results of this trial suggested improvement in maladaptive behavior on the ABC-C, CGI-I, and the Repetitive Behavior Scale in males with FXS and full methylation of *FMR1*, an outcome which was adequate to support ongoing development of AFQ056 for the treatment of FXS, with a larger multinational double-blind placebo-controlled 3-month trial evaluating the effects of multiple doses of the medication started in Fall of 2010 (clinicaltrials.gov). STX107 (Seaside Therapeutics) is completing phase I trials in healthy individuals and is expected to be ready for treatment trials in FXS in 2011 (clinicaltrials.gov). A double-blind placebo-controlled dose finding phase II trial of RO4917523 in adult males and females with FXS is underway and should be completed by late 2011 (clinicaltrials.gov). No serious safety concerns have yet emerged in any of these studies, although only small populations of individuals with FXS have thus far been exposed to mGluR5 blockers. It has been proposed that mGluR1 negative modulators might also be beneficial for individuals with FXS, but these have not been studied.

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* knockout mouse suggested promise of therapeutic benefit. Although lithium has been used for some time to treat mood instability and aggression in FXS (Hagerman et al. 2009; Berry-Kravis and Potanos 2004; Wang et al. 2010a), only anecdotal information on effectiveness was available prior to a pilot study initiated by Berry-Kravis et al. (2008a) to test the concept of inhibition of mGluR-activated translational signaling pathways as a treatment strategy for FXS by systematically exploring the effects of a short-term (2 months) treatment with lithium on a broad range of phenotypes, including behavior, cognition, and biophysical

measures, in a small cohort of subjects with FXS. In addition, since ERK was shown to be misregulated in the *fmr1* knockout and to have a reduced activation rate in lymphocytes from humans with FXS (Weng et al. 2008), ERK activation was explored as a potential biomarker for the effects of lithium on cellular signaling and more generally as a model for measuring changes in signaling during treatment with agents that may impact receptor-activated translational regulatory pathways. In this pilot open-label lithium trial in 15 patients with FXS, significant improvement in behavior was seen in on the Total Aberrant Behavior Checklist-Community Edition (ABC-C) Score, the Maladaptive Behavior subscore from the Vineland Adaptive Behavior Scale, a parent visual analog scale for target behaviors, and the CGI. Improvement in verbal memory on the RBANS List Learning task was also demonstrated in addition to normalization of abnormal ERK phosphorylation rates in lymphocytes (Berry-Kravis et al. 2008a). There were no major side effects, but polydipsia and polyuria were seen relatively frequently as expected, and there were a few subjects with abnormal thyroid measurements on lithium. These data suggested that further studies with a placebo-controlled trial would be indicated; however, such studies have not yet been carried out, partly due to concerns about chronic toxicity of lithium, but also related to hope that less toxic mechanism-based treatments will be available soon.

PAK inhibitors are being generated for possible use in humans with FXS (Drug Discovery: PAK Program), however these are still in preclinical development. PI3K inhibitors and GSK3β inhibitors have been proposed and used in the *fmr1* knockout, but are not yet in preclinical development for FXS.

Activity of individual proteins regulated by FMRP

Of synaptic protein targets thought to be regulated by FMRP and overexpressed in the *fmr1* knockout and, by extension, in FXS, only MMP9 has had a pharmaceutical inhibitor sufficiently well developed at present for human trials. Minocycline has been used in clinical trials to inhibit MMP9 in FXS. An open-label survey study suggested improvements in anxiety and language in patients with FXS treated in clinic with minocycline (Utari et al. 2010). An initial open-label trial of minocycline in 20 participants with FXS over age 12 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 due to elevated antinuclear antibodies 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 age 5 and up. The major detriment to using

minocycline in children <12 has been the likely side effect of yellow/brown discoloration of the permanent teeth. 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. The placebo-controlled trial should help delineate the true margin of benefit provided by minocycline to help with decisions, especially for younger children, about whether benefits of minocycline treatment exceed the impact of the tooth discoloration side effect.

Antibodies to reduce toxic Aβ metabolites of APP have already been piloted in human phase II trials in Alzheimer's disease (Frisardi et al. 2010). Such antibodies could also be used to reduce overexpressed Aβ in the human FXS brain, if justified by additional preclinical data in the knockout mouse.

Surface AMPA receptors and activity

A single human trial has been completed with CX516 (Cortex Phamaceuticals), a direct AMPA receptor-positive modulator or "ampakine" known to increase LTP and raise BDNF kevels, thus potentially increasing surface AMPA receptors (Jourdi et al. 2009). This was a double-blind placebo-controlled trial of effects of CX516 on safety and cognitive and behavioral efficacy measures carried out in a cohort of 49 individuals with FXS (Berry-Kravis et al. 2006). Conceptually, it was thought the CX516 would help compensate or correct the AMPA receptor deficit resulting from mGluR 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 show success in FXS; however, such molecules have not yet come to clinical trials.

Other receptors/proteins that regulate synaptic activity

Based on animal data discussed in the previous section, coupled with anecdotal clinical experience suggesting behavioral benefits from racemic baclofen and data from TMS studies demonstrating enhancement of cortical inhibition by racemic baclofen (McDonnell et al. 2007), a clinical trial of R-baclofen in humans with FXS has been conducted. R-baclofen is the enantiomer of racemic baclofen with more potent GABA-B agonist activity. The double-blind placebo-controlled crossover trial, conducted by Seaside Therapeutics, involved 4-week periods of placebo and active drug treatment for each subject. This trial showed benefit for R-baclofen over placebo in global preference for treatment period and clinician global impression, particularly evident in subgroups of FXS

patients with autism, more severe irritable behavior, or more severe social deficits. In the group with more impairment in social behaviors, improvements in the ABC Social Withdrawal Scale and Vineland Play and Leisure Scale were also seen (Wang et al. 2010c). There were no significant safety issues, and many subjects are continuing treatment through an extension study to evaluate long-term benefits and toxicity. Further development of R-baclofen is planned with additional clinical trials.

Other agents acting at an array of receptors have undergone an exploratory study in groups with FXS. These include donazepil, an anticholinesterase which raises acetyl choline in the brain and is extensively utilized for the maintenance of cognitive function in Alzheimer's disease. Donazepil showed promise for the treatment of behavior and social function in an open-label trial in participants with FXS and now is being studied in a larger placebo-controlled trial (clinicaltrials.gov). A small open-label study of memantine, an NMDA antagonist, in six individuals with FXS showed modest clinical benefit on a CGI in 4/6 patients, but no improvement on behavioral rating scales, and two patients developed significant irritability that limited treatment (Erickson et al. 2009). An open-label study of riluzole, a sodium channel blocker and glutamate uptake activator that indirectly decreases glutamate receptor activity, in five patients with FXS showed overall behavioral improvement in only one subject, although ERK activation rates normalized and there was a suggestion of improvement specifically in hyperactivity symptoms (Erickson et al. 2011). An anecdotal treatment experience has been reported with three adults with FXS treated with acamprosate, a drug approved for assisting with alcohol withdrawal that most likely interacts with multiple receptors but primarily may exert effects by acting as a mixed agonist/antagonist at NMDA receptors, activating GABA-A receptors and possibly inhibiting group I mGluRs. This case series observed improvement in language and behavior in all patients (Erickson et al. 2010a). One patient experienced limiting gastrointestinal side effects that are commonly seen with acamprosate. 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. Apipirazole has shown good success when used empirically in FXS clinic populations (Hagerman et al. 2009; Berry-Kravis and Potanos 2004), and resulted in improvement in ABC irritability, other ABC subscores and other behavioral rating scales in 15 individuals with FXS treated in a very recently completed open-label trial (Erickson et al. 2010b). Plans are underway to initiate a double-blind placebo-controlled trial of aripiprazole.

Translation of mRNAs regulated by FMRP using antisense technology

Treatments utilizing this strategy have just recently been initiated in preclinical animal models of FXS and are not in development yet for humans.

Trial design and hurdles to address

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 optimally demonstrate treatment effects in a clinical trial setting. These issues surrounding clinical trial design have not yet been worked out, nor are there models for cognitive treatment trials for any neurodevelopmental disorders. FXS, in fact, serves as a good model to develop such designs, particularly because FXS is a single genetic disorder in which all affected individuals have the same basic cellular defect as the primary cause of their brain disorder, a mouse model is available, some information on the mechanisms of the synaptic function of FMRP in brain is known, and aspects of FXS model more common disorders with likely mechanistic overlap, including autistic spectrum disorders, ADHD, and non-verbal learning disability.

Trial design issues that need to be resolved for each targeted treatment trial in FXS include: (1) length of placebo treatment and use of crossover designs or open-label extensions to ensure everyone gets a chance at drug and increase recruitment; (2) lack of information on optimal dosing and whether to determine this through dose escalation or flexible dosing within or between subjects or multiple fixed-dose arms; (3) how to best detect side effects in cognitively impaired individuals who may not be able to report symptoms accurately; (4) the most appropriate age range to study treatment effects, balancing concerns about safety that dictate adult trials first, with the possibility that rapid movement to younger ages may be needed despite minimal effects in older individuals because much more significant results may be seen by treating the underlying disorder in young children who are not as far along in the process of brain wiring and development and are still in school; (5) lack of understanding the length of treatment needed to impact brain wiring and demonstrate measurable cognitive improvement; (6) drug formulation and how to best deliver drug to younger children and individuals with oromotor dyspraxia; (7) inclusion of females and mosaic individuals, and whether to analyze response separately in these groups, as individuals with FMRP present in a fraction of cells may have different dosing ranges and differing toxicities; (8) whether to allow baseline medications and the balance between the need to analyze treatment effects in the

absence of medication interactions, problems with recruitment and patient discomfort if baseline medications have to be weaned, and the importance of demonstrating that new targeted treatments can actually improve symptoms even when treatment with the best available symptomatic regimen is already in place; (9) numbers of study visits and travel issues for a disorder in which subject numbers are limited and participants may need to come to trail sites from far away; and (10) the problem of lack of validated sensitive outcome measures for behavior or especially for cognition in FXS (or other developmental disorders) and lack of biomarkers known to correlate with functional improvement.

The design and evaluation of outcome measures for trials in FXS and neurodevelopmental disorders represents the most significant hurdle in trial design for targeted treatments. Choice of optimal outcome measures has been plagued by the need to test a broad ability range to prevent low- or high-functioning individuals from showing ceiling or floor effects, by problems with cooperation and variable performance, lack of knowledge about reproducibility of measures, and by the need to find measures that 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 be feasible and produce valid assessments (Berry-Kravis et al. 2006, 2008a, b, 2009). Only recently have investigators begun to
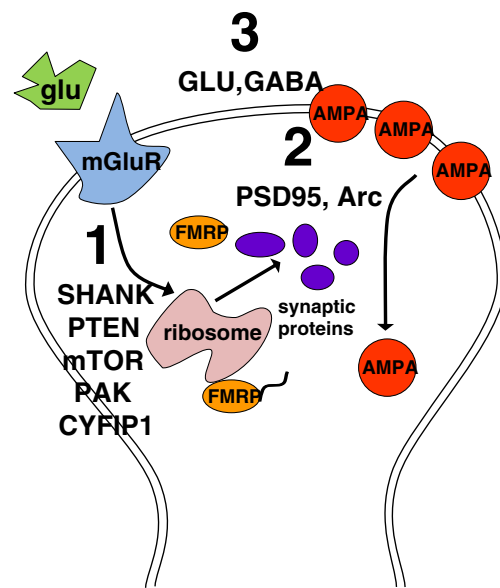
Fig. 2 Classes of potential overlap in synaptic mechanisms between ASD genes/proteins and pathways involved in FXS: (1) proteins involved in other forms of ASD may be in the signaling cascade for activation of FMRP-regulated translation; (2) FMRP may directly regulate proteins involved in different forms of ASD; and (3) convergence of glutamate and GABA pathway defects in FXS and ASD due to dysregulation of proteins generally important in maintaining inhibitory/excitatory balance and balance of activity in brain glutamate and GABA systems

develop templates for pre-trial feasibility, reproducibility, and validity assessment (Hessl et al. 2008a; Berry-Kravis et al. 2008b; Farzin et al. 2011; Knox and Berry-Kravis 2009). Choice of outcome measures must also balance the 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 (Hessl et al. 2008a; Farzin et al. 2011; Knox and Berry-Kravis 2009) that are more quantitative and may objectively measure core phenotypes and electrophysiology (such as eye tracking or PPI), thus advancing treatment science, but have no precedent for registration and are difficult to use to predict a specific functional outcome. Outcome measure development for targeted treatment trials in FXS has been a sufficiently difficult problem that a series of NIH meetings have been convened specifically to address this issue, with participation from the FDA (NIH 2009). These meetings have resulted in recommendations about best choice of currently existing measures, validation needs for existing measures, optimal types of measures to be developed, and work that needs to be done to develop them. No one behavioral scale was felt to capture the range and character of behaviors typically problematic in individuals with FXS, and development and validation of a fragile X-specific behavioral scale has been suggested. Initial work in preparation for the development of such a scale has been done by collecting ABC ratings from multiple sites and looking at items most endorsed for affected individuals with FXS relative to age and gender. This early work has indicated that the factor structure and items incorporated into the ABC (which was developed for individuals with general cognitive impairment and has been used extensively in autism) needs to be modified for optimal validity in FXS (Hessl et al. 2010).

Several years ago, the Fragile X Clinical and Research Consortium (FXCRC) was created with the help 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 multi-site clinical trials that will be necessary for FDA approval of targeted treatments.

Generalizing the FXS model to autism and other neurodevelopmental disorders

Clearly, there is overlap in the molecular and synaptic pathways between FXS and autism, and thus, targeted treatments for FXS will likely target dysregulated synaptic mechanisms in a subgroup of patients with autism and defects in the same pathways as are abnormal in FXS

(Fig. 2). Array studies in cohorts of patients with ASDs and mapping/mutation analyses in families segregating ASDs are generating a large list of genes implicated in autism which can be mapped onto specific brain pathways (Wang et al. 2010a; Marshall et al. 2008; Awadalla et al. 2010; Pinto et al. 2010). There are three broad categories of mechanistic overlap between ASD genes and pathways involved in FXS: (1) defects in proteins in the signaling cascade for the activation of FMRP-regulated translation such as SHANK, mTOR, PAK, and PTEN; (2) defects in proteins regulated directly by FMRP such as PSD95 and Arc; and (3) defects in proteins generally important in regulating activity levels and balance of activity in brain glutamate and GABA systems (Wang et al. 2010a). Treatments directed at all three of these mechanisms are becoming available in trials in FXS discussed above, and if successful, these treatment trials will likely be extended to cohorts with ASDs and other cognitive disorders. Progress in the development of these targeted treatments for FXS is likely to 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.

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