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Mechanism-based approaches to treating fragile X

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ABSTRACT

Fragile X is the leading inherited cause of mental retardation and autism. Recent advances in our mechanistic understanding of the disease have led to the identification of the metabotropic glutamate receptor (mGluR) as a therapeutic target for the disease. These studies have revealed that core defects in multiple animal models can be corrected by down regulation of mGluR5 signaling. Although it remains to be seen if mGluR5 antagonists or related approaches will succeed in humans with fragile X, the progress in fragile X stands as a strong testament to the power of applying knowledge of basic neurobiology to understand pathophysiology in a genetically validated model of human psychiatric disease. These breakthroughs and several of the resulting drug development efforts are reviewed.

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Contents

Abbreviations: Gp 1 mGluR, group 1 metabotropic glutamate receptor; FMRP, fragile X mental retardation protein; FX, fragile X; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NMDA, N-methyl-D-aspartate; PI, phosphoinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol, an endogenous activator of protein kinase C; IP_3, inositol-1,4,5-triphosphate; PI3K, phosphoinositide 3-kinase; Mnk, MAPK-interacting kinase; eIF4E, eukaryotic initiation factor 4E; 4E-BP, eIF4E binding proteins; CaM, calmodulin; ERK, extracellular signal-regulated kinase, also known as MAPK; eEF2K, eukaryotic elongation factor 2 kinase; eEF2, eukaryotic elongation factor 2; Arc, activity-regulated cytoskeleton-associated protein; MAP1B, microtubule-associated protein 1B; PLC, phospholipase C; mToR, mammalian target of rapamycin; DHPG, (*S*)-3,5-Dihydroxyphenylglycine, mGluR5 agonist; PSD, postsynaptic density; APP, amyloid precursor protein; GABA, gamma-aminobutyric acid; LTD, long-term depression; LTP, long-term potentiation; ODP, ocular dominance plasticity; MD, monocular deprivation; MD-RD, response depression after MD; MD-RP, response potentiation after MD; AGS, audiogenic seizure; IA, inhibitory avoidance learning; IAE, inhibitory avoidance extinction.

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1. Introduction

In 1943, Martin and Bell described a familial mental retardation syndrome with an X-linked inheritance pattern (Martin & Bell, 1943), known today as fragile X (FX) (Richards et al., 1981). FX is the most common inherited cause of mental retardation and autism. It is estimated to occur in 1:5000 males, and about half as many females (Coffee et al., 2009). Nevertheless, because the disease affects fewer than 200,000 people in the U.S., FX is considered a rare disease by the National Institutes of Health (NIH), and designated by the Food and Drug Administration (FDA) as an "orphan" indication (FDA, 2010). As a result, this disease has not, until recently, received much attention from large pharmaceutical companies looking to develop therapies.

Currently, there is no treatment for FX. The approach to patients focuses on managing symptoms using behavioral intervention and special education. Medications, which include antipsychotics, stimulants, and anticonvulsants (aimed at co-morbid aggression, attention deficit, and seizure respectively), are used cautiously, with recognition that this population may be especially sensitive to adverse effects of these drugs (see (Hagerman et al., 2009) for further review).

The recent surge in interest for developing FX therapies (NIH, 2010) stems from significant progress in our basic science understanding of the pathogenesis of the disease. Several breakthroughs—beginning with the identification of the gene that is disrupted in FX (Verkerk et al., 1991), development of a mouse model (Consortium, 1994), and identification of the metabotropic glutamate receptor 5 (mGluR5)-dependent plasticity phenotype (Huber et al., 2002), followed by the proposal of the "mGluR theory" (Bear et al., 2004), and culminating in the validation of this theory by genetic rescue of FX by mGluR5 knockdown (Dolen et al., 2007) as well as with pharmacologic blockade of mGluR5 (McBride et al., 2005; Yan et al., 2005; de Vrij et al., 2008)—have lead to the identification of a novel therapeutic target for FX. Here, we will review these advances and some of the resulting drug development efforts.

2. FMRP

In the majority of patients, FX is caused by a CGG repeat expansion in the *FMR1* gene on the X chromosome (which disrupts proper folding of the chromosome, making it susceptible to breaks, hence the name *fragile* X) (Verkerk et al., 1991). This mutation causes hypermethylation and transcriptional silencing of the gene, and the protein product, the fragile X mental retardation protein (FMRP), is not made (Fu et al., 1991; Pieretti et al., 1991). In a small minority of FX patients, a point mutation in the gene causes the disease by disrupting normal function of FMRP (De Boulle et al., 1993). Disease severity varies with the FMRP expression level, such that in females and mosaic males, who have some FMRP expression, the disease is relatively mild, while the most severe cases are males with a total absence of functional FMRP (De Boulle et al., 1993; Lugenbeel et al., 1995; Kaufmann et al., 1999; Tassone et al., 1999; Reiss & Dant, 2003; Loesch et al., 2004; Hatton et al., 2006). Accordingly, much of the early research on FX focused on understanding the cellular function of FMRP.

Studies of a de novo mutation in the I304N site of the *FMR1* gene (De Boulle et al., 1993; Feng et al., 1997a) and sequence homology analysis of FMR protein structure (Ashley et al., 1993) revealed the mRNA binding function of FMRP. FMRP has at least three mRNA binding domains: two K-Homology (KH) and one RGG box (see below for more on recently identified fourth domain (Bechara et al., 2009)). The I304N site encodes one of the two KH domains and the severity of the FX phenotype in the patient carrying this mutation suggests that RNA binding at this domain is critical to the biological function of FMRP (Feng et al., 1997a). Although KH domains are thought to recognize and competitively bind the so-called kissing complex (Darnell et al., 2005), natural mRNA ligands containing this structure have yet to be identified. The third RNA binding domain of FMRP is the RGG box, which recognizes stem-G-quartet loops found in several FMRP associated mRNAs (Schaeffer et al., 2001). Although point mutations highlighting the phenotypic significance of this domain have not yet been identified, FMRP-ligand mRNAs containing G quartets have been found (Brown et al., 2001; Darnell et al., 2001), and as we will see below, encode proteins that likely intersect with pathways relevant to the pathogenesis of the disease.

The mRNA binding capacity of FMRP suggested that FMRP might function to regulate the translation of mRNA to protein. Sucrose gradient fractionation studies showing association of FMRP with the sub-cellular translation machinery (ribosomes, polyribosomes, and granules) further implicated FMRP in protein synthesis regulation (Khandjian et al., 1995; Eberhart et al., 1996; Tamanini et al., 1996; Corbin et al., 1997; Feng et al., 1997a,b; Brown et al., 2001; Zalfa et al., 2003; Stefani et al., 2004; Aschrafi et al., 2005). However, these findings have not been without controversy.

The majority of fractionation studies have shown that FMRP co-sediments with polyribosomes and that this co-sedimentation is lost with I304N mutation, consistent with the idea that FMRP regulates protein synthesis by binding to actively translating mRNAs (Khandjian et al., 1995; Eberhart et al., 1996; Tamanini et al., 1996; Corbin et al., 1997; Feng et al., 1997a; Brown et al., 2001; Stefani et al., 2004). However, others have suggested an alternate mechanism whereby FMRP interacts with BC1 (a non-translatable RNA) to repress translation indirectly at the initiation step; this BC1–FMRP complex associates instead with monomeric 80s ribosomes and with mRNAs in the lighter fractions (and not polyribosomes) ((Zalfa et al., 2003; Bagni, 2008 but see (Stefani et al., 2004; Iacoangeli et al., 2008a,b)). Still others have shown that FMRP co-localizes with high-density granules (Aschrafi et al., 2005; Chen et al., 2008), which may represent large ribosomal aggregates whose translation has been stalled (Moult et al., 2006). While each co-sedimentation profile suggests a different mechanism by which FMRP might regulate protein synthesis, it is important to recognize that fractionation studies of this kind provide only indirect evidence that FMRP functions as a regulator of translation.

Direct evidence for FMRP's role in the regulation of protein synthesis has come from metabolic labeling experiments that measure translation in the presence and absence of functional FMRP both *in vitro* (Laggerbauer et al., 2001; Li et al., 2001; Dolen et al., 2007) and *in vivo* (Qin et al., 2005a). These studies have consistently shown that FMRP acts to inhibit protein synthesis. For example, Li et al. (2001) examined the effect of recombinant FMRP on translation in rabbit reticulocyte lysate (RRL) and showed an FMRP dose-dependent suppression of translation of brain RNA. This suppressive effect was abolished by competitive inhibition of FMRP and by removal of FMRP-binding sequences from mRNA transcripts, providing evidence for the specificity of the interaction between FMRP and the template mRNA (Li et al., 2001). This effect was shown to be due specifically to translation suppression rather than increased mRNA degradation, since exposure of mRNAs to FMRP in the absence of the translation machinery (from RRL) produced no decrease in template levels (Li et al., 2001). Using the same RRL assay as well as microinjected Xenopus oocytes, Laggerbauer et al. (2001) have also shown a negative regulatory role for FMRP. In addition, repression of translation is lost in FMRP with the I304N mutation in both systems, consistent with the idea that this domain confers translation regulatory function to the protein (Laggerbauer et al., 2001).

Dolen et al. (2007) have examined the role of FMRP in regulating protein synthesis in a more intact *in vitro* preparation, using ^{35}S-methionine/cystine for metabolic labeling of hippocampal brain slice. These studies showed that *Fmr1* knockout (KO) mice (Consortium, 1994) have approximately 20% increased hippocampal protein synthesis compared to wild-type (WT), again consistent with a negative regulatory role for FMRP. Furthermore, electrophoretic separation of radiolabeled translation products showed that this increase in the *Fmr1* KO is not limited to one or few predominant protein species, but rather extends across a broad range of resolved molecular weights (Dolen et al., 2007).

Finally, Qin et al. (2005a) have examined a protein synthesis regulatory role for FMRP *in vivo*. WT and *Fmr1* KO mice were catheterized and injected with an intravenous pulse of radioactive leucine. Autoradiograms of radioactively labeled sections of the brain were then quantified by brain region and genotype. A number of brain regions showed an increase in protein synthesis in the *Fmr1* KO versus WT brain, again consistent with FMRP's function as a negative regulator of translation. Importantly, these results proved that disruptions of protein synthesis in the *Fmr1* KO mouse are globally manifested in the brain and measurable in the intact animal, raising the possibility that measurements of protein synthesis could serve as a biomarker of disease (Bishu et al., 2008, 2009). Indeed, studies are currently under way to test this hypothesis in human patients with FX (NIH, 2010).

3. Metabotropic glutamate receptor signaling

Meanwhile, as the aforementioned studies began to reveal the function of FMRP, significant advances were being made towards understanding the mechanisms of glutamatergic signaling and synaptic plasticity. Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Glutamatergic signaling is mediated by both ionotropic and metabotropic receptors at the synapse. The ionotropic glutamate receptors (iGluRs) include alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors (Monaghan et al., 1985; Cincotta et al., 1989). As shown in Fig. 1, the metabotropic glutamate receptors (mGluRs) are a family of eight receptors categorized into three groups (Group 1, 2 and 3) based on their sequence homology, agonist and antagonist pharmacology, and coupling to signal transduction pathways (Houamed et al., 1991; Masu et al., 1991; Abe et al., 1992; Nakanishi, 1992; Tanabe et al., 1992; Saugstad et al., 1994; Conn & Pin, 1997).

Group 1 mGluRs (Gp1 mGluRs), which are further subdivided into mGluR1 and mGluR5 subtypes, couple to Gq-like G-proteins, signal through activation of phospholipase C (PLC), and are primarily postsynaptic (Romano et al., 1995; Lujan et al., 1996). In contrast, Groups 2 and 3 mGluRs couple to Gi-like G-proteins, signal through adenylate cyclase, and are mostly presynaptic (Conn & Pin, 1997). The two Gp1 mGluRs show complementary expression patterns, with mGluR5 expression highest in the hippocampus, neocortex, and striatum (but notably absent in the cerebellum), whereas mGluR1 expression is largely restricted to the cerebellum (Shigemoto, 2000). For the purposes of the current discussion, special attention will be given to mGluR5 because the synaptic signaling mechanisms relevant to the pathogenesis of FX have been most extensively elucidated in the hippocampus and neocortex. However, because the mechanistic approach to cerebellar phenotypes has also been validated for mGluR1, the role of these receptors in the pathogenesis of the disease will be briefly addressed at the end of the discussion.

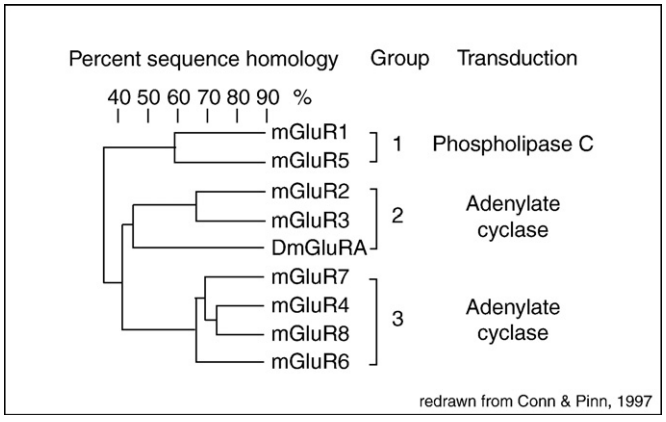

Fig. 1. Dendrogram and pharmacologic classification of mammalia(mGluR1-8) and *Drosophila* (DmGluRA) mGluRs.

Although mGluR5 is canonically defined by its coupling to PLC transduction, as shown in Fig. 2, more recent evidence suggests that receptor signaling occurs through at least three distinct cascades. (1) The canonical PLC cascade: phosphoinositol (PI) hydrolysis results in the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2) into two second messengers: diacylglycerol (DAG) (an endogenous activator of protein kinase C) and inositol-1,4,5-triphosphate (IP_3) (which mobilizes receptor-mediated release of intracellular stores of Ca^{2+}) (Abe et al., 1992; Pin et al., 1992; Joly et al., 1995; Watabe et al., 2002). (2) The PI3K/Akt/mTOR cascade: phosphorylation of the phosphoinositide 3-kinase (PI3K) activates Akt, which turns on the mammalian target of rapamycin (mToR) (Hou & Klann, 2004; Antion et al., 2008; Ronesi & Huber, 2008). (3) The extracellular signal-regulated kinase (ERK) cascade: the tyrosine kinase Src phosphorylates and activates MEK, which in turn phosphorylates and activates ERK (also called microtubule-associated protein kinase, MAPK) (Ferraguti et al., 1999; Choe & Wang, 2001; Thandi et al., 2002; Berkeley & Levey, 2003; Adwanikar et al., 2004; Gallagher et al., 2004; Mao et al., 2005; Grueter et al., 2006; Garcia et al., 2008).

Interestingly, also shown in Fig. 2, all three pathways have been directly or indirectly linked to the regulation of protein synthesis (Gallagher et al., 2004; Hou & Klann, 2004; Klann & Dever, 2004; Banko et al., 2006; Davidkova & Carroll, 2007; Proud, 2007; Antion et al., 2008; Park et al., 2008; Waung et al., 2008). For example, activated mToR phosphorylates 40S ribosomal protein S6 kinase, which in turn phosphorylates ribosomal protein S6 (Hou & Klann, 2004; Klann & Dever, 2004; Banko et al., 2006; Antion et al., 2008). ERK activates the MAPK-interacting kinase (Mnk), which leads to phosphorylation of the eukaryotic initiation factor 4E (eIF4E) (Banko et al., 2006). Both activated mToR and ERK phosphorylate eIF4E binding proteins (4E-BPs) (Klann & Dever, 2004). Furthermore, stimulation of mGluR5 can also induce translational de-repression at synapses by inducing CYFIP1 dissociation from eIF4E, allowing for translation, perhaps through an interaction with the Rac1 GTPase pathway (not shown in Fig. 2) (Napoli et al., 2008). Together, these pathways when activated initiate 5′ cap-dependent translation of mRNAs (Klann & Dever, 2004; Proud, 2007). Most recently, calcium-dependent dissociation of eukaryotic elongation factor 2 kinase (eEF2K) from mGluR5 and phosphorylation of eukaryotic elongation factor 2 (eEF2) by activated calcium–calmodulin (Ca^{2+}-CaM)/eEF2K have been implicated in rapid translation of the activity-regulated cytoskeleton-associated protein (Arc) (Park et al., 2008; Waung et al., 2008) and microtubule-associated protein 1B (MAP1B) (Davidkova & Carroll, 2007) in response to activation of mGluR5. Although these experiments did not specifically address the interaction between the eEF2K and PLC signaling, a reasonable (but speculative) source for the second messenger calcium implicated in eEF2K signaling is that which derives from coupling to PLC, since it is known that activation of the PI hydrolysis by PLC results in IP3-dependent release of calcium from intracellular stores (Abe et al., 1992; Joly et al., 1995; Nakamura et al., 1999; Watabe et al., 2002; Dudman et al., 2007; Hong & Ross, 2007). Together, these results suggest that binding of glutamate to mGluR5 activates multiple intracellular second messenger cascades that regulate protein synthesis. Furthermore, mGluR5 activation recruits mRNAs to the actively translating polyribosome fraction (Muddashetty et al., 2007), induces protein synthesis (Weiler & Greenough, 1993; Weiler et al., 1997; Job & Eberwine, 2001; Todd et al., 2003; Davidkova & Carroll, 2007; Mameli et al., 2007; Muddashetty et al., 2007; Westmark & Malter, 2007; Park et al., 2008; Waung et al., 2008), and, as detailed below, many of the long-term consequences of mGluR5 activation are protein synthesis-dependent.

4. Metabotropic glutamate receptor-dependent synaptic plasticity

Activity-dependent synaptic plasticity is defined as any long-lasting form of synaptic modification (strengthening or weakening) that is synapse specific and is induced by specific patterns of pre- and/or postsynaptic firing (Hebb, 1949; Bienenstock et al., 1982; Bear et al.,

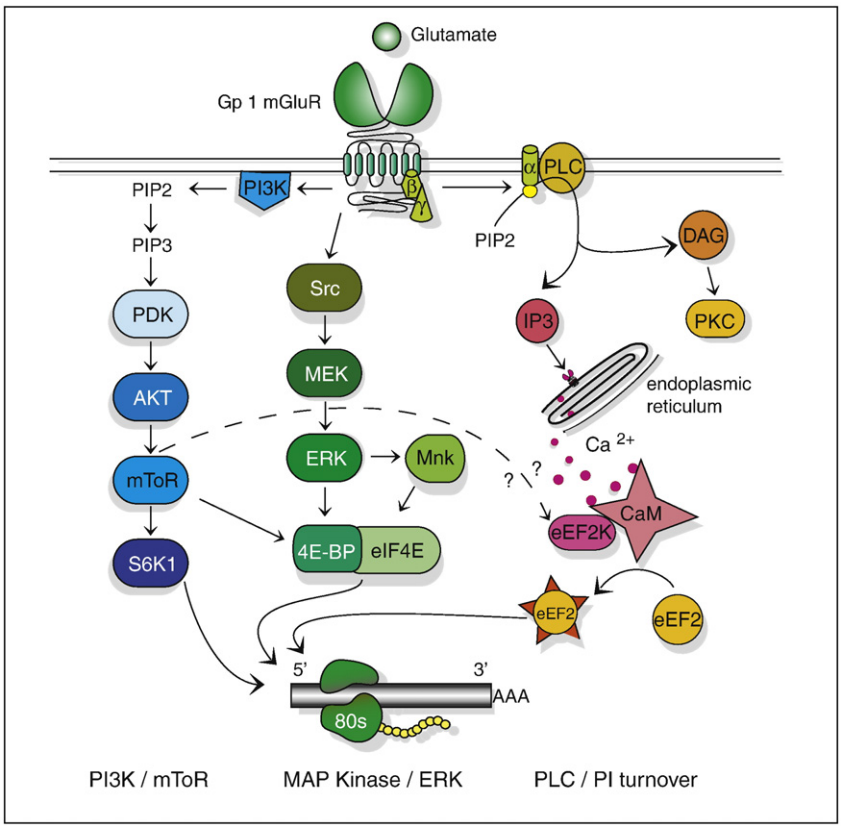

Fig. 2. Signaling cascades coupling to mGluRs (PI3K, MAPK, PLC) converge to regulate protein synthesis.

1987). It manifests as electrophysiological, molecular, and morphological changes, and it provides the basis for most models of learning and memory as well as the development of response selectivity and cortical maps.

Synaptic plasticity can be triggered experimentally, producing long-term potentiation (LTP) and long-term depression (LTD) of the synapse, using a variety of induction protocols, including: theta-burst stimulation (TBS), high-frequency stimulation (HFS), low-frequency stimulation (LFS), paired-pulse LFS (PP-LFS), chemical (direct receptor activation without afferent stimulation), and pairing protocol (afferent stimulation paired with postsynaptic depolarization), to name a few. While early mechanistic studies of LTP and LTD focused on the role of the NMDA receptor-mediated induction, mGluR-mediated forms have also been identified (Linden et al., 1991; Bolshakov & Siegelbaum, 1994; Oliet et al., 1997; Huber et al., 2000; Karachot et al., 2001). Furthermore, it has now become clear that neurons are highly variable in terms of the type of LTP and LTD they express. Thus, when describing these phenomena, it is necessary to define the specific synapse and circuit, the phase (induction, expression, and maintenance), and the developmental epoch (including the recent history of synaptic activity) that is being studied (see (Malenka & Bear, 2004) for review). For the purposes of the current discussion, we will focus on the hippocampal CA1-Shaffer collateral synapse because this is a well-characterized circuit for mGluR5-mediated LTD, with the caveat that alternate mechanisms for induction, expression, and maintenance may exist elsewhere in the brain.

mGluR5-dependent forms of LTD can be induced in hippocampal area CA1 by stimulation of Schaffer collaterals with PP-LFS or by chemical induction (by application of the specific mGluR5 agonist (*S*)-3,5-Dihydroxyphenylglycine, DHPG) (Bolshakov & Siegelbaum, 1994; Oliet et al., 1997; Schnabel et al., 1999; Huber et al., 2000; Fitzjohn et al., 2001; Huber et al., 2001; Wu et al., 2004; Volk et al., 2006; Volk et al., 2007). Hippocampal DHPG-LTD is occluded by PP-LFS, is NMDA receptor independent, and is absent in mGluR5 KO mice, consistent with the idea that this form of synaptic plasticity represents a distinct cellular mechanism of synaptic modification mediated by mGluR5 (Huber et al., 2001). Furthermore, since mGlu5 receptors are concentrated in an annulus around the postsynaptic density (PSD) where they are stimulated only under conditions of high glutamate release at the synapse (Lujan et al., 1996; Kennedy, 2000), it seems likely that plasticity induced by mGluR5 subserves specific neuronal functions encoded by distinct patterns of synaptic activity (Huber et al., 2000; Huber et al., 2001).

Induction of mGluR-LTD in hippocampal CA1 requires translation but not transcription, since protein synthesis inhibitors like cyclohexamide and anisomycin, but not the transcription inhibitor actinomycin, prevent the induction of paired-pulse- and DHPG-induced LTD (Huber et al., 2000, 2001; Hou & Klann, 2004). Furthermore, an injection of cap analogue m^7GppG into the postsynaptic cell blocks DHPG-LTD in CA1, indicating a postsynaptic locus for protein synthesis dependence ((Huber et al., 2000) that is it is developmentally regulated (see Nosyreva & Huber, 2005). Postsynaptic coupling of mGluR5 to intracellular signal transduction pathways that regulate translation, described above, represents a likely mechanism for the protein synthesis dependence of hippocampal mGluR-LTD. For example, inhibitors of ERK block mGluR-LTD, and DHPG treatment of hippocampal slice induces phosphorylation of ERK (Gallagher et al., 2004) as well as ERK-dependent phosphorylation of Mnk1, eIF4E, and 4EBP (Banko et al., 2006). Consistent with these biochemical findings, increasing eIF4E complex availability by genetic elimination of 4E-BP2 increases the magnitude of DHPG-LTD in an ERK-dependent manner (Banko et al., 2006). Inhibition of the PI3K/Akt/mToR pathway has also been shown to block mGluR-LTD (Banko et al., 2006; Ronesi & Huber, 2008).

Finally, RNAi knockdown of eEF2K in neuronal culture as well as transgenic eEF2K KO in hippocampal slices, blocks DHPG-LTD, consistent with the role of this signal transduction cascade in mediating the protein synthesis dependence of hippocampal mGluR-LTD (Davidkova & Carroll, 2007; Park et al., 2008; Waung et al., 2008). However, given the previous reports of the calcium independence of DHPG-LTD in the

hippocampus (Schnabel et al., 1999; Fitzjohn et al., 2001), the proposed role of Ca^{2+} in regulating this cascade (Ca^{2+} dynamically modulates the interaction of eEF2K with mGluR5 and eEF2K is a Ca^{2+}/CAM dependent kinase) (Park et al., 2008), may not be the whole story. Interestingly, eEF2K also contains a putative Homer binding site (Park et al., 2008) that is phosphorylated by multiple signaling kinases, including PI3K/mToR/S6K, ERK, and PKA (Browne & Proud, 2002). Furthermore, Homer scaffolding proteins link mGluR5 to the PSD as well as to signaling partners like IP_3R (Tu et al., 1999) Erk (Mao et al., 2005) and PI3K/Akt/mToR (Ronesi & Huber, 2008). Disruption of this interaction between Homer and mGluR5 blocks mGluR-LTD, mGluR-mediated protein synthesis, and activation of PI3K/Akt/mToR pathways (Ronesi & Huber, 2008).

At this time, it is unclear how each of these signaling cascades is specifically recruited to encode presumptively distinct neuronal functions. Nevertheless, as noted above, they each converge to initiate mGluR5-dependent translation and are available at the postsynaptic compartment (Asaki et al., 2003), further highlighting the importance of local protein synthesis for the induction of mGluR-LTD.

Expression of mGluR-LTD in the hippocampus occurs by regulation of AMPA receptor (GluR1 and GluR2 subunits) trafficking (Snyder et al., 2001; Xiao et al., 2001; Watabe et al., 2002; Nosyreva & Huber, 2005; Grooms et al., 2006; Huang & Hsu, 2006; Moult et al., 2006; Nosyreva & Huber, 2006; Volk et al., 2006; Davidkova & Carroll, 2007; Park et al., 2008; Waung et al., 2008) as well as by changes in the biophysical properties of AMPA receptors (Delgado and O'Dell, 2005; Huang & Hsu, 2006; Moult et al., 2006). When it has been examined, this process has been shown to require the synthesis of new proteins at the synapse: DHPG-induced AMPA receptor internalization and LTD are abolished by translation inhibitors (Snyder et al., 2001; Nosyreva & Huber, 2005, 2006) as well as by blockade of eEF2K-dependent Arc protein synthesis (Park et al., 2008; Waung et al., 2008) or MAP1B (Davidkova & Carroll, 2007).

Thus far, the discussion of mGluR-LTD has focused on mechanisms for the initial decrease in synaptic strength lasting 30–60 min. However, perhaps of greater significance are the processes that allow this form of LTD to last hours, days, or even weeks. Structural remodeling of the synapse has long been hypothesized to be the mechanism for long-term maintenance of plasticity (see (Alvarez & Sabatini, 2007) for review). The dendritic spine is the postsynaptic site of excitatory transmission in the mammalian brain; its volume is linearly correlated the surface area of the postsynaptic density (PSD) it contains (Freire, 1978; Spacek & Hartmann, 1983; Harris & Stevens, 1989), and PSD surface area is linearly correlated with AMPA receptor content of the synapse (Nusser et al., 1998). Thus, regulation of glutamatergic receptor trafficking, required for the expression of synaptic plasticity (see above, and Malinow & Malenka, 2002), is thought to be the preamble to morphologic changes in spine number, shape, and size.

Several studies have shown that NMDA-LTP induction produces an increase in the size of potentiated dendritic spines (Engert & Bonhoeffer, 1999; Matsuzaki et al., 2001; Lang et al., 2004; Matsuzaki et al., 2004; Yang et al., 2008), whereas NMDA-LTD induction results in a decrease in dendritic spine head size (Chen et al., 2004; Nagerl et al., 2004; Zhou et al., 2004; Bastrikova et al., 2008; Yang et al., 2008). Recently, a promising mechanistic link between AMPA receptor delivery and structural growth of the dendritic spines during NMDA-LTP has been proposed. Following LTP induction, recycling endosomes carrying AMPA receptors, as well as other synaptic proteins and machinery, translocate into dendritic spines where they exocytose their cargo and fuse with the membrane, thereby simultaneously increasing dendritic spine size and delivering AMPA receptors (Park et al., 2006). It remains to be seen whether similar mechanisms are employed for LTD-induced AMPA receptor internalization and spine size reduction. This mechanism of structural plasticity is mediated by NMDA receptor-dependent Ca^{2+} signaling coordinated by myosin Vb (MyoVb), a Ca^{2+} sensitive motor (Wang et al., 2008). However, it is unclear whether these mechanisms will be employed for the maintenance of mGluR5-dependent synaptic plasticity. Another member of this motor protein family, myosin Va (MyoVa), is recruited by a synaptic stimulation with DHPG for localization of TLS, an mRNA binding protein, and its ligand mRNAs into dendritic spines (Yoshimura et al., 2006), raising the possibility that these motor proteins may play a role in mGluR5-mediated plasticity.

While the structural plasticity studies described above have examined the role of NMDA receptor-dependent forms of LTP and LTD, changes in spine size have also been observed with the application of DHPG to hippocampal neurons in culture (Vanderklish & Edelman, 2002). Furthermore, like DHPG-LTD induction (Huber et al., 2000; Hou & Klann, 2004; Banko et al., 2006; Davidkova & Carroll, 2007; Park et al., 2008; Waung et al., 2008) and DHPG-induced AMPA receptor internalization (Snyder et al., 2001; Nosyreva & Huber, 2005, 2006; Davidkova & Carroll, 2007; Park et al., 2008), this process is protein synthesis-dependent (Vanderklish & Edelman, 2002). There is also evidence that both ERK (Kornhauser & Greenberg, 1997; Wu et al., 2001; Goldin & Segal, 2003; Zhou et al., 2004) and mToR signaling cascades regulate synaptic remodeling (Jaworski et al., 2005; Kumar et al., 2005; Jaworski & Sheng, 2006), although these studies did not specifically address coupling to mGluR5 transduction. Taken together, these results support the idea that morphological changes at the level of dendritic spines provide a potential structural basis of how DHPG-induced decreases in synaptic strength are made long lasting, and that this process, like mGluR-LTD induction and expression, is protein synthesis-dependent.

The discussion thus far has focused on mGluR-LTD, but mGluRs mediate other forms of synaptic plasticity as well. For example, mGluR5 has been implicated in a phenomenon known as metaplasticity (the plasticity of plasticity); at this level, plasticity is not expressed as a change in synaptic strength but instead as a change in the ability to induce subsequent synaptic plasticity (Abraham & Bear, 1996). For example, the activation of mGluRs facilitates the subsequent induction of NMDA receptor-dependent LTP (Cohen & Abraham, 1996; Cohen et al., 1998; Raymond et al., 2000; Stoop et al., 2003; Bortolotto et al., 2005, 2008; Ayala et al., 2009) and inhibits subsequent induction of NMDA receptor-dependent LTD (Harney et al., 2006; Ayala et al., 2009; Ireland & Abraham, 2009). The activation of mGluR5 has also been implicated in late-phase LTP (L-LTP) (Francesconi et al., 2004) and synaptic depotentiation (Zho et al., 2002) both of which are protein synthesis-dependent. These findings may reflect the fact that mGluR5 activation regulates NMDA receptor trafficking (Snyder et al., 2001; Watabe et al., 2002; Ireland & Abraham, 2009) and function (Alagarsamy et al., 2001). As new synaptic plasticity induction protocols are developed, it is likely that other forms of mGluR-mediated synaptic plasticity will be discovered. For now it seems clear that many forms require protein synthesis for their induction, expression, and perhaps also maintenance.

5. Metabotropic glutamate receptor-mediated plasticity *in vivo*

With the expanse of scientific literature dedicated to LTP and LTD, it can be easy to overlook the fact that these experimental paradigms were originally developed to model intrinsic patterns of neuronal activity responsible for naturally-occurring experience-dependent plasticity and learning in the intact animal (Bliss & Lomo, 1973; Ito & Kano, 1982). Before their discovery, landmark studies of *in vivo* ocular dominance plasticity (ODP) in monkeys and cats had already established a role for experience-dependent modifications in shaping the circuitry of the brain during development (Hubel & Wiesel, 1964, 1970; Hubel et al., 1977). The advent of transgenic technologies (Mansour et al., 1988) and adaptation of the ODP paradigm to rodents (Drager, 1978; Gordon & Stryker, 1996; Porciatti et al., 1999; Prusky & Douglas, 2003; Sawtell et al., 2003; Frenkel & Bear, 2004), has made it possible to return to this *in vivo* plasticity paradigm, revealing shared mechanisms between ODP and LTP/LTD (see Smith et al., 2009 for review).

Establishing a role for mGluR5 in mediating these developmental processes in visual cortex has been more difficult. Original studies posited

a role for mGluR5 based on the observation that the developmental decline in glutamate-stimulated PI turnover is correlated with the decline in experience-dependent synaptic plasticity in visual cortex (Dudek & Bear, 1989; Dudek et al., 1989). This proposition was bolstered by studies showing the laminar redistribution of mGluR5 expression in the visual cortex across development (with the highest density of layer 4 expression corresponding to the height of the critical period) (Reid et al., 1995, 1997). Arguing in favor of a role for mGluR-mediated synaptic plasticity in this brain region, studies using the mGluR antagonist (+/)-alpha-methyl-4-carboxyphelylglycine (MCPG), showed a blockade of LTD (Haruta et al., 1994; Kamishita et al., 1995) and depotentiation (Hensch & Stryker, 1996) induced with LFS in visual cortical slice. Infusion of MCPG into kitten visual cortex *in vivo*, however, did not disrupt visual cortical plasticity (expressed as a change in contralateral/ipsilateral evoked unit response ratio) induced by monocular deprivation (MD), which led these authors to argue for dissociation between synaptic plasticity processes (mGluR-LTD/depotentiation) and ODP (Hensch & Stryker, 1996). Subsequent studies, however, demonstrated that although MCPG does competitively block actions of the synthetic glutamate agonist 1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid (ACPD), it is ineffective against specific metabotropic actions of glutamate (PI turnover) in visual cortex (Huber et al., 1998). Significantly, coupling to PI turnover is necessary for the induction of LTD in the visual cortex (Choi et al., 2005).

Recently, molecular genetic knockdown of mGluR5 (Jia et al., 1998) has allowed for the re-examination of the role of mGluR5 in ODP (Dolen et al., 2007). As referred to above, ODP can be assessed in mice using visually evoked potentials (VEPs) recorded in layer 4 of visual cortex (the site of primary thalamocortical inputs) (Porciatti et al., 1999), and it has been correlated with behavioral measures of visual acuity (Prusky & Douglas, 2003). Chronic VEP recordings allow for the measurement of field potentials from each eye (contralateral and ipsilateral to the recording electrode), both before and after MD by monocular lid suture. In juvenile WT mice, 3 days of MD induces contralateral-eye response depression (MD-RD), whereas 7 days of MD induces ipsilateral-eye response potentiation (MD-RP) (Frenkel & Bear, 2004). In contrast, in mGluR5 heterozygous mice at this age, 3 days of MD induces neither MD-RP (normal) nor MD-RD (abnormal) (Dolen et al., 2007), implicating mGluR5 in the modulation of this form of plasticity. Although it is tempting to draw parallels between mGluR5-mediated MD-RD and mGluR-LTD, it is important to recognize that mechanisms of LTD and ODP vary across visual cortical layers (Daw et al., 2004; Crozier et al., 2007; Liu et al., 2008), and to date, only in layer 2/3 of visual cortex have there been pharmacologically reliable descriptions of mGluR-LTD (Choi et al., 2005). Future studies examining LTD in layer 4 of visual cortex in mGluR5 KO mice will therefore be instructive. Furthermore, although both protein synthesis (Taha & Stryker, 2002) and ERK (Di Cristo et al., 2001; Takamura et al., 2007) are also required for ODP, at this time it is merely speculative to suggest coupling to mGluR5-mediated plasticity (but see below for further discussion).

6. A role for mGluRs in regulating the inhibitory excitatory balance

Agonists of Gp 1 mGluRs act as convulsants in rodents (Tizzano et al., 1995; Conn & Pin, 1997). Conversely selective Gp 1 mGluR antagonists block seizures in some (Thomsen et al., 1994; Tizzano et al., 1995; Chapman et al., 2000; Borowicz et al., 2004; Zadrozniak et al., 2004; Lojkova & Mares, 2005; Yan et al., 2005; Jesse et al., 2008; Mares, 2009; Pacey et al., 2009) but not all (Zadrozniak et al., 2004; Lojkova & Mares, 2005; Loscher et al., 2006; Olive & Becker, 2008; Witkin et al., 2008) rodent models of epilepsy. Interestingly, mGluR5 antagonists appear to be more effective as anticonvulsants during development (through postnatal week 3 in rodents) (Lojkova & Mares, 2005; Mares, 2009), and ineffective for example in controlling seizure during ethanol withdrawal (Olive & Becker, 2008), whose pathogenesis is thought to be NMDA receptor-mediated (Nagy, 2008). Thus the variation in anticonvulsant activity likely reflects the heterogeneity of seizure etiologies and the specificity of mGluR5 receptor blockade as a mechanism of action.

Consistent with this idea, mGluR5 stimulation induces epileptiform discharges in the hippocampus, a process that is protein synthesis-dependent (Merlin et al., 1995; Merlin & Wong, 1997; Merlin et al., 1998; Wong et al., 1999; Lee et al., 2002). In addition, there is a growing evidence that mGluR-LTP between inhibitory (GABAergic) and excitatory (glutamatergic) neurons, serves to regulate the inhibitory–excitatory balance in the brain (see (Anwyl, 2009) for review). Moreover, stimulation of mGluR5 with DHPG induces mobilization of mRNA encoding GABA-Aδ receptors to dendrites (Dictenberg et al., 2008). Whether this up-regulation reflects homeostatic or homosynaptic changes is unclear at this time. Nevertheless, taken together, these results suggest that mGluR-mediated modulation of the inhibitory–excitatory balance is sensitive to the state of mGluR5-dependent protein synthesis (Stoop et al., 2003).

7. The mGluR theory of fragile X

Human patients with FX have significant cognitive impairments, with mental retardation in the moderate-to-severe range, as well as behavioral problems, dysmorphic features, and seizure disorder (Hagerman & Hagerman, 2002). As reviewed above, synaptic plasticity is the foundation of most theories of learning and memory and cognitive development; therefore early studies of the pathogenesis of the disease examined the *Fmr1*KO mouse model for possible disruptions of plasticity. However, investigations of NMDA receptor-dependent forms of plasticity failed to reveal a deficit in *Fmr1* KO mice (Godfraind et al., 1996; Paradee et al., 1999; Huber et al., 2002). At around the same time, a novel form of LTD in the hippocampus had just been characterized. As discussed earlier, this form of LTD is induced by activation of mGluR5 and is normally protein synthesis-dependent (Huber et al., 2000, 2001). Because stimulation of mGluR5 with DHPG had been shown to lead to synaptosomal translation of FMRP (Weiler et al., 1997), it seemed possible that FMRP was a downstream protein necessary for the induction, expression, or maintenance of mGluR-LTD, which led to the prediction that mGluR-LTD would be deficient in *Fmr1* KO mice. Instead, mGluR-LTD was significantly exaggerated in *Fmr1* KO mice (Huber et al., 2002).

At the same time, studies of the function of FMRP had begun to reveal its role as a negative regulator of protein synthesis, likely through its interaction with polyribosomes (see discussion above). In neurons, this translation machinery can be found at the base of dendritic spines where it has long been thought to exert local control over synaptic function (Steward & Schuman, 2001). Furthermore, recall that synaptic mGluR5 activation induces protein synthesis (Weiler & Greenough, 1993; Weiler et al., 1997; Job & Eberwine, 2001; Todd et al., 2003; Davidkova & Carroll, 2007; Mameli et al., 2007; Muddashetty et al., 2007; Westmark & Malter, 2007; Park et al., 2008; Waung et al., 2008) and many of the long-term consequences of Gp1 mGluR activation are protein synthesis-dependent (Huber et al., 2000, 2001; Karachot et al., 2001; Snyder et al., 2001; Vanderklish & Edelman, 2002; Gallagher et al., 2004; Hou & Klann, 2004; Banko et al., 2006; Davidkova & Carroll, 2007; Mameli et al., 2007; Pfeiffer & Huber, 2007; Park et al., 2008; Ronesi & Huber, 2008; Waung et al., 2008). Taken together, these findings suggested the possibility that Gp1 mGluRs and FMRP might work in functional opposition to regulate mRNA translation at the synapse, and that in the absence of FMRP, unchecked mGluR-dependent protein synthesis leads to the pathogenesis of the disease (Bear et al., 2004). This so called "mGluR theory" (see Fig. 3) has now been tested directly, and as we will see next, these and other results overwhelmingly support the use of Gp1 mGluR antagonists in the treatment of FX.

8. Validation of the mGluR theory

As noted above, previous attempts to characterize mGluR-mediated process have been confounded by off-target and non-specific effects of

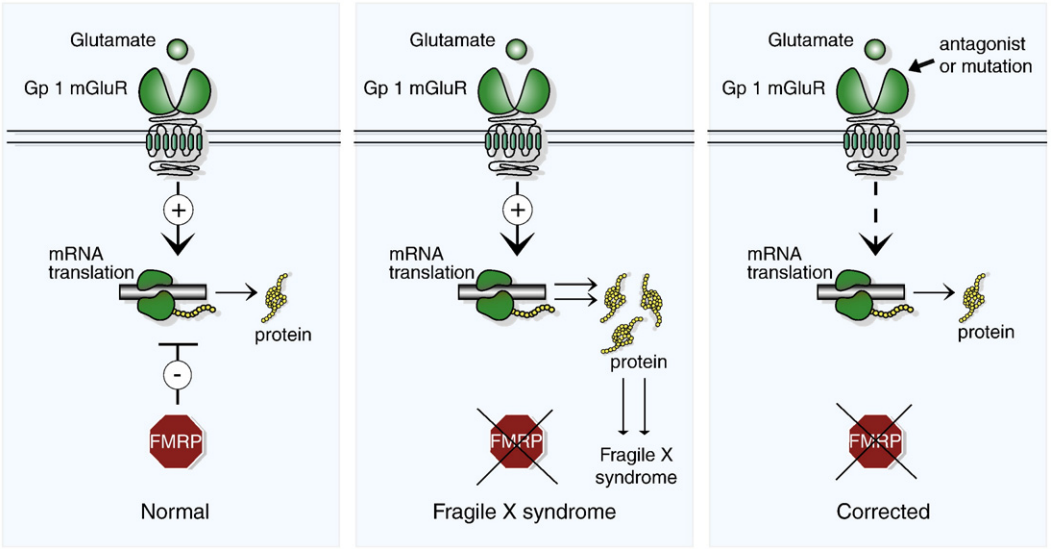

Fig. 3. The mGluR theory: Opponent regulation of protein synthesis by FMRP and group I mGluRs, is disrupted in the absence of FMRP. Reduction of mGluR signaling restores the balance and corrects FX phenotypes.

pharmaceuticals aimed at these receptors. A method that avoids such confounds is genetic analysis, because here the contribution of a gene (or protein product) is determined by preventing (in the case of knockout) or reducing (in the case of knockdown) expression of target proteins. Genetic interaction experiments represent a further iteration of this strategy and are accomplished by generating crosses between mutants (e.g., double mutants) to determine the contribution of multiple genes to a single phenotype. Taking advantage of this powerful tool has made it possible to test the mGluR theory directly, by generating double mutants of *Fmr1* and *Grm5* (the gene that encodes mGluR5) and examining multiple phenotypes relevant to the pathogenesis of FX (Dolen et al., 2007). Importantly, these experiments were carried out in mice, which unlike fruit fly (*Drosophila melanogaster*), carry the full complement of mammalian mGluRs. Because the fly ortholog of mGluR (DmGluR) couples to adenylate cyclase (making it more similar to Group 2 mGluRs, see Fig. 1), and because unlike in mammalian brain, acetylcholine (ACh), not glutamate, is used as the primary excitatory neurotransmitter in the central nervous system (Su and O'Dowd, 2003; Lee and O'Dowd, 1999), this model organism has limitations for testing the role of Gp1 mGluRs in the pathogenesis of FX (Dolen & Bear, 2005).

8.1. Correction of protein synthesis and mGluR-LTD phenotypes

In these studies, the increased hippocampal protein synthesis phenotype seen in *Fmr1* KO mice (described above), was restored to WT levels by a 50% reduction of mGluR5 expression in the *Fmr1* KO/*Grm5* heterozygote cross (CR) (Dolen et al., 2007). Consistent with genetic interaction between FMRP and mGluR5, there was no evidence (from electrophoretic separation) that these proteins regulate different subsets of translation products. Furthermore, because the *Grm5* heterozygote (Het) did not itself show decreased protein synthesis, these results argue against the null hypothesis (i.e., no genetic interaction, return to WT levels reflects the average of unrelated changes in opposite directions). The lack of a protein synthesis phenotype in Het mice also suggests that a therapeutic dose of an mGluR5 antagonist for FX patients should not have negative side effects in unaffected individuals. As noted above, protein synthesis measurements are currently being developed as a biomarker of disease in human patients with FX (NIH, 2010), so it is encouraging to see that this phenotype responds to manipulation of mGluR5 signaling in the *Fmr1* KO model of the disease.

The exaggerated mGluR-LTD phenotype seen in *Fmr1* KO mice is likely to reflect the increased basal rate of protein synthesis because in these animals, the dependence on protein synthesis is lost (Hou et al., 2006; Nosyreva & Huber, 2006; Park et al., 2008). The functional consequence of 50% reduction in mGluRs is a concomitant reduction in the magnitude of DHPG-LTD in CR compared to *Fmr1* KO mice, which parallels the return to WT levels of protein synthesis in CR mice (Dolen et al., 2007). Together, these results suggest that the over-produced proteins in the *Fmr1* KO are able to maintain mGluR-LTD in the absence of further protein synthesis. Consistent with this interpretation, Arc protein has been reported to be constitutively up-regulated in *Fmr1* KO brain ((Zalfa et al., 2003) but see Park et al., 2008), and DHPG stimulation induces rapid synthesis of Arc protein in WT but not *Fmr1* KO mice (Park et al., 2008; Waung et al., 2008). As noted above, both knockdown and acute blockade of new Arc synthesis prevent mGluR-LTD and AMPAR trafficking in WT mice (Waung et al., 2008). Consistent with Arc's role as an "LTD-expression protein," AMPA receptor internalization is also exaggerated in *Fmr1* KO mice (Nakamoto et al., 2007). Because *Fmr1*/*Arc* double KO mice show incomplete impairment of mGluR-LTD (Park et al., 2008), these studies reveal that other overproduced proteins must be available to sustain mGluR-LTD in the *Fmr1*KO hippocampus.

Indeed, compelled by the explanatory power of the mGluR theory, a number of studies have identified other mRNA targets of FMRP regulated by mGluR5 including: APP, CaMKII, eEF1A, GluR1/2, MAP1B, PSD95, SAPAP 3/4, RGS-5, GABA-A ((Brown et al., 2001; Darnell et al., 2001; Schaeffer et al., 2001; Miyashiro et al., 2003; Sung et al., 2003; Todd et al., 2003; Kindler et al., 2004; Antar et al., 2005; Huang et al., 2005; Hou et al., 2006; Bramham & Wells, 2007; Davidkova & Carroll, 2007; Muddashetty et al., 2007; Narayanan et al., 2007; Westmark & Malter, 2007; Zalfa et al., 2007; Dictenberg et al., 2008; Menon et al., 2008), reviewed by Bassell (Bassell & Gross, 2008; Bassell & Warren, 2008)). Many of these mRNAs have been shown to be elevated in the polyribosome fraction at the basal state in the *Fmr1*KO and insensitive to further DHPG-induced-recruitment into the actively translating fraction (Muddashetty et al., 2007).

Consistent with the genetic rescue of the exaggerated protein synthesis phenotype (Dolen et al., 2007), several studies have reported basal-state elevations of MAP1B and CaMKII protein in *Fmr1* KO brain (Zalfa et al., 2003; Hou et al., 2006, Lu et al., 2004 #91), which occlude further DHPG-induced translation of these proteins (Hou et al., 2006; Muddashetty et al., 2007). Similarly, the amyloid precursor protein (APP) as well as two of its degradation products, $A\beta_{40}$ $A\beta_{42}$, are basally elevated in *Fmr1* KO and occlude DHPG-mediated up-regulation of APP in synaptoneurosomes (Westmark & Malter, 2007). Although PSD-95 protein levels are not basally elevated in *Fmr1* KO brain, DHPG-induced translation of PSD-

95 protein (Todd et al., 2003; Muddashetty et al., 2007) and PSD-mRNA stabilization (Zalfa et al., 2007) seen in WT are also absent in the *Fmr1* KO brain. Each of these proteins has variously been implicated in the induction, expression, and maintenance of LTD (Snyder et al., 2001; Delgado and O'Dell, 2005; Hsieh et al., 2006; Davidkova & Carroll, 2007; Mameli et al., 2007; Xu et al., 2008), so future studies examining their contribution to the exaggerated mGluR-LTD phenotype in FX will be informative. Finally, PP2A and S6K1 have been implicated in DHPG-mediated phosphoregulation of FMRP as a repressor of translation (Narayanan et al., 2007, 2008). Whereas these findings provide another mechanistic link between mGluR5 and FMRP in the regulation of protein synthesis, the role of this putative link in WT mGluR-LTD or expression of exaggerated mGluR-LTD in *Fmr1* KO is unknown at this time.

8.2. Correction of dendritic spine phenotype

One of the hallmark features of FX in humans is the overabundance of long thin spines (Hinton et al., 1991; Irwin et al., 2001). This phenotype is recapitulated in the *Fmr1* KO mouse (Comery et al., 1997; Nimchinsky et al., 2001; Galvez & Greenough, 2005; McKinney et al., 2005; Dolen et al., 2007; Pfeiffer & Huber, 2007; de Vrij et al., 2008; Bilousova et al., 2009) and parallels the protein synthesis-dependent increase in the density of long thin spines seen in response to stimulation with DHPG (Vanderklish & Edelman, 2002). To test the opponent regulatory role for mGluR and FMRP, genetic interaction studies have examined the dendritic spine density phenotype, revealing complete rescue of the *Fmr1* KO phenotype by 50% reduction of mGluR5 signaling in both apical and basal dendrites of layer 2/3 visual cortical pyramidal neurons (postnatal day 30) (Dolen et al., 2007). Again arguing against the null hypothesis and verifying the therapeutic value, 50% reduction of mGluR5 signaling had no effect on spine morphology on its own (Dolen et al., 2007). This rescue of the dendritic spine morphology has recently been replicated using mGluR5 antagonists (MPEP and fenobam) in hippocampal cell culture (de Vrij et al., 2008). If AMPA receptor internalization is indeed the preamble to morphologic remodeling of the synapse (see discussion above), the dendritic spine phenotype in FX is consistent with exaggerated DHPG-mediated, protein synthesis-dependent AMPA receptor internalization (Snyder et al., 2001; Nosyreva & Huber, 2005, 2006; Davidkova & Carroll, 2007; Nakamoto et al., 2007; Park et al., 2008) and the loss of AMPA receptors in the cerebral cortex of *Fmr1* KO mice (Li et al., 2002). The genetic rescue of this phenotype, as well as the protein synthesis and mGluR-LTD phenotypes, provides further evidence for a common mechanistic underpinning for the pathogenesis of FX (Dolen et al., 2007).

8.3. Correction of in vivo plasticity phenotypes

As discussed above, studies have revealed common mechanisms between ODP and synaptic plasticity, providing *in vivo* evidence for the functional importance of these processes shaping the circuitry of the brain during development. Their importance is further highlighted by the discovery of their role in the pathogenesis of FX, a neurodevelopmental disorder (Dolen et al., 2007). These studies revealed that *Fmr1* KO mice have a hyper-plastic ODP profile. Recall that juvenile WT mice show MD-induced response depression (MD-RD) after 3 days of monocular lid suture, and MD-induced response potentiation (MD-RP) after 7 days of monocular lid suture (Frenkel & Bear, 2004). In the *Fmr1* KO mouse, the plasticity profile is characterized by both MD-RD (normal) and MD-RP (abnormal) after 3 days of MD by lid suture. This is in one sense the opposite of the hypo-plastic profile seen in *Grm5* HT mice, which have neither MD-RD (abnormal) nor MD-RP (normal) after 3 days of MD. Reduction of mGluR5 signaling by 50% restores the *Fmr1* KO plasticity profile to WT levels, arguing in favor of genetic interaction between mGluR5 and FMRP (Dolen et al., 2007). Indeed, because FMRP is a negative regulator of translation, these findings lend further support to the protein synthesis dependence of ODP (Taha & Stryker, 2002), perhaps mediated by mGluR5 coupling to ERK (Di Cristo et al., 2001). As mentioned above, further study *in vitro* is necessary to shed light on the synaptic mechanisms of this interaction. For now, note that like the exaggerated mGluR-LTD phenotype in hippocampus, the ODP phenotype in the visual cortex reflects exaggerated plasticity in *Fmr1* KO and is corrected by reduction of mGluR5 expression.

Although *Fmr1* KO and *Grm5* HT phenotypes represent in some sense opposite plasticity profiles, it is unlikely that the genetic rescue in the CR mice reflects the average of unrelated changes in opposite directions, because this interpretation would predict compound effects of phenotypes, resulting in a plasticity profile characterized by both MD-RP (*Fmr1* KO phenotype) and absence of MD-RD (*Grm5* HT phenotype). Instead, CR mice have normal MD-RD and normal absence of MD-RP after 3 days of monocular deprivation (Dolen et al., 2007). These results provide further evidence for the therapeutic value of targeting mGlu5 receptors for the treatment of FX. Nevertheless, the presence of an ODP phenotype in *Grm5* HT mice raises the concern that these drugs may have unwanted side effects in unaffected controls that will need to be monitored when establishing therapeutic doses.

8.4. Correction of learning and memory phenotype

Humans with FX show mental retardation in the moderate-to-severe range, so identifying a cognitive behavioral phenotype in FX has been a priority. Nevertheless, until recently, studies of cognitive performance in *Fmr1* KO mice have revealed only subtle deficits (Consortium, 1994; Kooy et al., 1996; D'Hooge et al., 1997; Paradee et al., 1999; Dobkin et al., 2000; Musumeci et al., 2000; Peier et al., 2000; Van Dam et al., 2000; Yan et al., 2004; Koekkoek et al., 2005; Qin et al., 2005b; Zhao et al., 2005; Brennan et al., 2006) (see for review Bernardet & Crusio, 2006). Taking into consideration the proposed role of Gp1 mGluRs and FMRP in regulating translation, genetic interaction studies examining a protein synthesis-dependent form of hippocampal learning and memory—inhibitory avoidance extinction (IAE) (Power et al., 2006)—have revealed an interesting *Fmr-1* KO phenotype (Dolen et al., 2007). In these animals, inhibitory avoidance (IA) learning, which is mediated by NMDA receptor-dependent LTP (Whitlock et al., 2006), is not different from WT (Consortium, 1994; Dolen et al., 2007) but may be strain specific (see Qin et al., 2005b). The extinction of this memory (IAE) is exaggerated in *Fmr1* KO mice (Dolen et al., 2007), and is reminiscent of the exaggerated mGluR-LTD phenotype in the hippocampus of *Fmr1*KO mice (Huber et al., 2002). Furthermore, it is corrected by reducing mGluR5 expression by 50%, providing further evidence of the therapeutic value of targeting this receptor for treatment of the cognitive phenotype in FX (Dolen et al., 2007). Once again the *Grm5* HT mice showed no impairments, arguing against the null hypothesis and providing further evidence for the safety of targeting this receptor.

8.5. Correction of seizure phenotype

The most common neurological abnormality in FX is epilepsy, which has an estimated incidence of 15–20% (Musumeci et al., 1988; Hecht, 1991; Kluger et al., 1996; Musumeci et al., 1999; Berry-Kravis, 2002). Seizures present as both partial seizure (specific focus at onset) and generalized seizure (diffuse whole brain involvement from onset) (Berry-Kravis, 2002). Patients lose consciousness during seizure (complex seizure), and when generalized in form, the seizure episode is characterized by convulsions (e.g., tonic–clonic) (Musumeci et al., 1999). If uncontrolled, status epilepticus (SE) is a rare but fatal clinical outcome. Finally, the age of onset of these seizures is 2 years, and in most cases, seizures and epileptiform activity resolve by puberty (Berry-Kravis, 2002). The pathogenesis of this phenotype in FX is unknown, but it is interesting to note that patients show hypersensitivity to acoustic stimuli (Miller et al., 1999) and elevated evoked responses to these stimuli in cortex (Castren et al., 2003).

The epilepsy phenotype is recapitulated in the *Fmr1* KO mouse model of the disease. Susceptibility to audiogenic seizures (AGS) is

consistently and significantly higher in *Fmr1*-KO mice compared to WT littermates (Musumeci et al., 2000; Yan et al., 2004, 2005; Dolen et al., 2007; Pacey et al., 2009). Audiogenic seizures are generalized, convulsive (tonic–clonic), complex, and evoked by a loud (125 dB SPL at 0.25 m) auditory stimulus. Similar to epilepsy in human FX, they are present early in postnatal development and resolve by post-adolescence (Yan et al., 2005). In addition, hypersensitivity to auditory stimuli leads to a lower seizure threshold in *Fmr1*-KO mice (Chen & Toth, 2001), and FMRP expression itself is modulated in response to sensory stimulation and seizure induction *in vivo* (Irwin et al., 2000; Todd & Mack, 2000).

Because Gp1 mGluRs have been implicated in modifying seizure susceptibility, and because this process is developmentally regulated and protein synthesis-dependent (see discussion above), genetic interaction studies sought to examine the specific interaction between mGluR5 and FMRP in regulating the process (Dolen et al., 2007). These studies have shown that reduction of mGluR5 expression significantly reduces seizure incidence in *Fmr1* KO mice (Dolen et al., 2007). Although the rescue in this case was incomplete, these results nevertheless implicate mGluRs in the pathogenesis of the seizure phenotype in FX and raise the possibility that insufficient reduction of mGluR5 gene dosage accounts for the incomplete rescue. This interpretation is consistent with studies using the mGluR5 antagonist MPEP, which completely blocks the AGS phenotype in a dose-dependent manner (Yan et al., 2005), and *in vitro* studies showing increased mGluR5-mediated epileptiform discharges in hippocampus of *Fmr1* KO mice (Chuang et al., 2005). Recalling the mGluR-stimulated synthesis of APP and the increased levels of this protein in the *Fmr1*KO (Westmark & Malter, 2007), the mGluR-mediated mechanism of pathogenesis is also consistent with the increased seizure susceptibility seen in mice over-expressing APP, a phenotype that is exacerbated in the FRAXAD double mutant (lacking FMRP and over-expressing APP) (Westmark et al., 2008).

Several studies have implicated a disruption of GABAergic signaling in the pathogenesis of the epilepsy phenotype in FX. For example, GABA-A receptor expression (El Idrissi et al., 2005), particularly the delta subunit (D'Hulst et al., 2006), is reportedly decreased in the *Fmr1* KO, and the GABA-Aδ mRNA is a ligand of FMRP (Miyashiro et al., 2003; Dictenberg et al., 2008). Furthermore, electrophysiological studies have suggested that *Fmr1* KO mice have decreased GABAergic inhibitory tone in the subiculum (Curia et al., 2009) and striatum (Centonze et al., 2008) despite normal basal synaptic transmission, excitability, and paired-pulse facilitation in the CA1 region of the hippocampus (Godfraind et al., 1996; Paradee et al., 1999; Huber et al., 2002). Interestingly, mGluR5 agonists stimulate the mRNA transport of GABA(Aδ) to dendrites (Dictenberg et al., 2008), and this response is absent in *Fmr1* KO mice. As discussed earlier in the context of exaggerated mGluR-LTD, this phenotype in the *Fmr1* KO could represent occlusion of mGluR5-mediated stimulation, leading to the over-production of GABA-Aδ receptor proteins. However, the results described above suggest the opposite change in GABA-A receptor expression (El Idrissi et al., 2005; D'Hulst et al., 2006; Centonze et al., 2008; Curia et al., 2009). There are several potential explanations to resolve this paradox: (1) The mGluR-stimulated recruitment of GABA-Aδ mRNA to dendrites is a homeostatic adaptation to constitutive hyperexcitability (mediated by mGluR5-stimulated synthesis of other proteins (Chuang et al., 2005)), and this response is depleted rather than occluded in the *Fmr1* KO. (2) Recent reports of FMRP as a positive modulator of translation (Bechara et al., 2009) raise the possibility that GABA-Aδ mRNA is a ligand for FMRP translational activation, in which case constitutive mGluR-stimulated recruitment of GABA-Aδ mRNA to dendrites could lead to reduced GABA (Aδ) receptor protein expression the *Fmr1* KO. (3) The mixed phenotype reflects the complex interplay of circuit-level changes, including those mediated by mGluR-LTP at inhibitory (GABAergic) synapses (Anwyl, 2009) as well as the contribution of G-protein (Gi/o)-coupled GABA-B receptors.

Indeed, the GABA-B receptor agonist arbaclofen (also called R-baclofen) has very recently been shown to attenuate the AGS phenotype in *Fmr1* KO mice (Pacey et al., 2009). These results are complicated by the reduced seizure susceptibility in the *Rgs4*/*Fmr1* double-knockout mouse (Pacey et al., 2009), because *Rgs4* encodes regulator of G-protein signaling 4 (RSG4), an inhibitor of Gp1 mGluRs (Saugstad et al., 1998). However, more recently studies showing additional RGS4 coupling to GABA-B receptors and inward rectifying K^+ channels (KIR) (Fowler et al., 2007) suggest that the attenuation of the phenotype may reflect the contribution of GABA-B receptors. Furthermore, the GABA-B receptor antagonist CGP 46381 exacerbates the AGS phenotype in double-knockout mice but not in WT mice (in WT, only co-administration of both CGP 46381 and the mGluR agonist CDPPB induces AGS) presumably because mGluR5-dependent signaling is already elevated in the double KO but not WT mice (Pacey et al., 2009). Together with the *Fmr1*/*Grm5* genetic interaction studies (Dolen et al., 2007), these results suggest that the AGS phenotype in FX reflects a contribution of exaggerated signaling through mGluR5 and reduced signaling through GABA-B receptors. Future studies examining the interplay between these two G-protein signaling cascades will therefore be informative.

8.6. Somatic phenotypes: correction of growth abnormality but not macroorchidism

Fragile X boys and girls show an increased rate of growth, as measured by height, during the preadolescent period (Loesch et al., 1995). The hypothalamus is an integral part of the hypothalamic–pituitary–adrenal (HPA) axis that controls endocrine function. The lateral and ventromedial hypothalamus are thought to be important for feeding behaviors: lesions of these regions lead to anorexia and obesity, respectively, in mice (Bellinger & Bernardis, 2002). Moreover, both the ventromedial and the lateral hypothalamus have high levels of mGluR5 expression (van den Pol et al., 1995). In addition, mGluR5 is a regulator of feeding behavior, and mGlur5 antagonists are known to be appetite suppressants (Bradbury et al., 2005). This growth abnormality is recapitulated in the *Fmr1* KO mouse, and although it is subtle (20% at its maximum), pre-pubertal increases in body weight are apparent (Dolen et al., 2007). Furthermore, genetic interaction studies reveal that mGluR HT mice have normal body weight, as do *Fmr1* KO animals, with a 50% reduction in the mGluR5 expression (Dolen et al., 2007). Although the mechanism of this rescue is not clear, this evidence points to clear genetic interaction between FMRP and Grm5, perhaps at the level of the HPA axis, in regulating body weight. Furthermore, these results provide evidence that somatic phenotypes regulated in the brain can be rescued by targeting mGluR5. As noted before, the absence of a phenotype in the HT argues for a true genetic interaction and for the safety of this manipulation in unaffected controls.

The macroorchidism phenotype (large testicles) has been recognized for over 20 years and occurs in over 80% of adult males with FX (Nielsen et al., 1982). Nevertheless, this phenotype is not specific to FX; it is estimated to occur in nearly 30% of the mentally retarded male population, and FX accounts for only a small subset of these cases (Primrose et al., 1986; Hagerman et al., 1988). These estimates preceded identification of the mutation in FX, so the diagnosis of the disease is uncertain. Both the phenotype itself and the developmental profile are recapitulated in the *Fmr1* KO mouse model (Kooy et al., 1996; Peier et al., 2000; Dolen et al., 2007). Gp 1 mGluR RNAs are abundantly expressed in the testicles, with high levels of both mGluR5 and mGluR1 expression in the seminiferous tubuli and germ cells and high mGluR1 expression in the Sertoli cells (Storto et al., 2001). These expression studies raise the intriguing possibility that the pathogenesis of the macroorchidism phenotype in FX is related to exaggerated Gp1 mGluR signaling. Despite these correlations, genetic interaction studies failed to reveal evidence for mGluR5's involvement in the pathogenesis of this phenotype. Even when mGluR5 was completely knocked out, the phenotype persisted on the *Fmr1* KO background, showing that the absence of rescue was not related to mGluR5 gene

dosage. Nevertheless, both *Grm5* HT and KO mice showed normal testicular weight, indicating that reduction of mGluR5 signaling should have no adverse effects in unaffected patients. Because the other Gp1 mGluR—mGluR1—is also highly expressed in the testicles, the possibility remains that this is the relevant receptor for the pathogenesis of the macroorchidism phenotype.

8.7. Cerebellar phenotypes: the role of mGluR1

The mGluR theory applies to both Gp1 mGluRs—mGluR5 and mGluR1 (Bear et al., 2004). However because mGluR1 expression is largely restricted to the cerebellum (Shigemoto, 2000), the discussion thus far has focused on mGluR5. Nevertheless, impairments in cerebellar function are likely relevant to the clinical presentation of FX, because these patients are known to have deficits in coordination and motor function (Hagerman & Hagerman, 2002; Koekkoek et al., 2005). As discussed above, Gp1 mGluR-LTD was initially described at the cerebellar parallel fiber (PF) – Purkinje cell (PC) synapse (Linden et al., 1991) and later confirmed to be mGluR1-dependent (Aiba et al., 1994). Like hippocampal mGluR5-dependent plasticity at the CA1–Shaffer collateral synapse, mGluR1-LTD at the cerebellar PF-PC synapse is protein synthesis-dependent (Karachot et al., 2001). Moreover, this form of synaptic plasticity is also exaggerated in the *Fmr1* KO mouse (Koekkoek et al., 2005), consistent with an opponent regulatory role of mGluR1 and FMRP (Bear et al., 2004; Huber, 2006).

Although it is unknown at this time whether the long-term expression mechanism of PF-PC mGLuR1-LTD involves remodeling of dendritic spines, as in the neocortex and hippocampus, dendritic spines on cerebellar Purkinje cells in the *Fmr1* KO mice are elongated (Koekkoek et al., 2005). These findings suggest that morphological consequence of mGluR1 activation in the cerebellum recapitulates morphological plasticity in response to activation of mGluR5 in the hippocampus (Vanderklish & Edelman, 2002) and that likewise, this process is exaggerated in FX.

Finally, eyeblink conditioning is a form of classical conditioning that has been used to study cerebellar learning and memory. This behavioral paradigm is thought to require mGluR1-LTD because the LTD is impaired in mGluR1 KO mice (Aiba et al., 1994) and is necessary for computational models of this behavior (Medina et al., 2000). Interestingly, eyeblink conditioning is impaired in both *Fmr1* KO mice and human patients with disease (Koekkoek et al., 2005), once again linking mGluR1 to the pathogenesis of a cerebellar phenotype in FX (Bear et al., 2004; Huber, 2006). Together, these findings implicate mGluR1 as a viable therapeutic target for FX.

9. Clinical trials

Transgenic animal models of Mendelian single gene disorders provide the opportunity to interrogate molecular pathophysiology associated with the clinical diseases. In particular, the effects of the fragile X mutation on brain development and function have been facilitated by the generation of *Fmr1* KO animal models. As reviewed above, the accumulated evidence suggests that mGluR5 is a valid target for development of drugs to treat FX. The ultimate goal of molecular medicine—translation of basic science discoveries into novel therapeutics—may soon be realized for FX. Several companies are currently assessing the hypothesis that selective antagonism of mGluR5 signaling will provide therapeutic benefit for humans with FX.

9.1. Therapies targeting mGluR5

Pharmaceutical interest in inhibitors of mGluR5 stems from preclinical research suggesting potential therapeutic utility for a variety of human conditions including anxiety (Nordquist et al., 2008), convulsions (Moldrich et al., 2003), pain (Walker et al., 2001), depression (Brodkin et al., 2002), Parkinson's disease(Breysse et al., 2002) and gastroesophageal reflux disease (GERD) (Frisby et al., 2005). Recently, Addex Pharmaceuticals has demonstrated efficacy in man for migraine and GERD in preliminary, proof-of-concept studies (Addex Pharmaceuticals Inc: Addex Pharmaceuticals first half 2007 financial results. Press Release (2007) July 25).

Neuropharm Group PLC recently reported results of a single-dose trial with the selective mGluR5 antagonist fenobam in 12 subjects with FX (Berry-Kravis et al., 2009). Single doses up to 150 mg were well tolerated. Although the study was not powered to assess efficacy, some trends for beneficial clinical effects were reported. Of note, the anxiolytic efficacy of fenobam was originally assessed in clinical trials performed from 1978 to 1982 (Itil et al., 1978; Pecknold et al., 1980; Lappierre & Oyewumi, 1982; Pecknold et al., 1982), but development was discontinued. Recently, Porter et al. (2005) discovered fenobam to be a selective mGluR5 antagonist. Because safety and tolerability of fenobam in humans had been previously established, it was possible to rapidly open an IND and initiate clinical trials in subjects with FX. Although fenobam is a selective mGluR5 antagonist *in vitro*, it is rapidly metabolized and has variable systemic bioavailability *in vivo*. Furthermore, although efficacy comparable to other mGluR5 antagonists has been demonstrated in typical preclinical anxiety models, the atypical behavioral disruption observed with fenobam suggests either significant off-target activity or formation of a biologically active metabolite. Plasma levels of fenobam are highly variable in humans after oral dosing and are not correlated with efficacy (Itil et al., 1978; Berry-Kravis et al., 2009). Thus, it is not clear at this time whether additional clinical trials with fenobam will provide a thorough test of the mGluR theory in people with fragile X.

In addition, Novartis has completed a phase-2 fragile X trial in Europe with its mGluR5 modulating compound (AFQ56), although the results of this trial have not yet been published (http://www.clinicaltrials.gov/ct2/show/NCT00718341?term=fragile+X&rank=7). Finally, most recently (in November 2009) Roche has initiated a phase-2 fragile X trial in the U.S. with its mGluR5-modulating compound (RO4917523) with an estimated study completion date of June 2010 (http://clinicaltrials.gov/ct2/show/NCT01015430?term=fragile+x+roche&rank=1).

9.2. Therapies targeting GABA-B

Drugs that inhibit glutamate release have the potential to reduce excessive mGluR-mediated protein synthesis. For example, GABA-B receptor agonists inhibit presynaptic release of glutamate, postsynaptic transmission and intracellular signaling downstream from mGluR5 (Scanziani et al., 1992; Isaacson & Hille, 1997; Sohn et al., 2007) and therefore may indirectly inhibit signaling through mGluR5 receptors. As predicted, the selective GABA-B agonist baclofen attenuates the AGS phenotype observed in *Fmr1* KO mice (Pacey et al., 2009).

The R-isomer of baclofen (arbaclofen) is known to be a more potent GABA-B agonist than the S-isomer (Hill & Bowery, 1981) and is reported to be more potent in a variety of preclinical biologic and behavioral assays (Olpe et al., 1978; Johnston et al., 1980; Haas et al., 1985; Fattore et al., 2002). Seaside Therapeutics initiated a trial in November 2008 to assess safety, tolerability, and efficacy of STX209 (arbaclofen) in subjects with fragile X (NIH, 2010). This placebo controlled, double-blind, crossover study will evaluate the safety, tolerability, and efficacy of 28 consecutive days of dosing of STX209 in 60 subjects with FX. The primary efficacy outcome measure is the irritability subscale of the Aberrant-Behavior Checklist score (http://clinicaltrials.gov/ct2/results?term=arbaclofen+fragile+x).

10. Development of novel mGluR5 antagonists

The potent mGluR5 antagonist STX107 came from a portfolio of mGluR5 compounds discovered by scientists at Merck & Co., Inc. A number of studies conducted at Merck highlighted the compound's

desirable drug-like properties, as well as the ability to penetrate the blood–brain barrier and bind mGluR5 receptors in the brain. After Seaside Therapeutics in-licensed the compound and demonstrated its efficacy in animal models of FX, it was then subjected to the battery of tests necessary to enable the filing of an investigational new drug (IND) application with the FDA. Included in those studies were *in vitro* studies such as genotoxicity assays (Ames test and chromosome aberration assay) and studies to address effects on an important cardiac ion channel (hERG). *In vivo*, dose-ranging and repeated-dose good laboratory practice (GLP) toxicology studies were conducted in rats and primates. The rat toxicology study design also incorporated an *in vivo* genotoxicity component (*in vivo* micronucleus assay) as well as a CNS safety study. Independently, cardiovascular (primate), respiratory (rat), and gastrointestinal (rat) safety studies were conducted. Seaside Therapeutics conducted these studies under a cooperative translational research grant with the NIH. Results from all studies were evaluated, the results were compiled in a full IND document that was approved by the FDA, and Phase 1 trials in normal human volunteers are ongoing (http://clinicaltrials.gov/ct2/show/NCT00965432?term=fragile+x&rank=20). A favorable profile of STX107 in normal human volunteers will allow progression into studies in subjects with FX.

While the majority of advanced compounds of current interest are the result of historical drug discovery programs, next-generation mGluR5 antagonists continue to be pursued. For example, Seaside Therapeutics is partnering with the Vanderbilt Program in Drug Discovery at Vanderbilt University Medical Center to discover novel mGluR5 antagonists. Initial efforts focused on high-throughput screening and targeted combinatorial chemistry to generate new compounds. The goal of this collaboration is to not only focus on the use of these antagonists but also to incorporate new biological insights into the disease, thus translating new knowledge and novel compounds into potential effective treatments.

11. Conclusions

The human genome was sequenced on the promise that understanding the genetic basis for disease would point the way to fundamentally new treatments. FX stands poised to fulfill this promise of "molecular medicine". Although it remains to be seen if mGluR5 antagonists or related approaches will succeed in humans with FX, it is now well established that core defects in multiple animal models can be corrected by down regulation of mGluR5 signaling. The progress in FX research and treatment stands as a strong testament to the power of applying knowledge of basic neurobiology to understand pathophysiology in a genetically validated model of human psychiatric disease.

Conflict of interest

G. Dölen — no conflict.

R. Carpenter, T. Ocain, M. Bear — financial interest in Seaside Therapeutics.

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