Developing BACE-1 inhibitors for FXS

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INTRODUCTION

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and the leading known genetic cause of autism (Wang et al., 2010). Clinical features include moderate to severe intellectual disability, autistic-like behavior, anxiety, seizures, and macroorchidism (Hagerman et al., 2009). Neuroanatomical features include an overabundance of long, thin tortuous postsynaptic spines (Beckel-Mitchener and Greenough, 2004). In the majority of cases, FXS is caused by a trinucleotide repeat expansion in the promoter region of the fragile X mental retardation 1 (FMR1) gene, which leads to promoter methylation and lack of translation of fragile X mental retardation protein (FMRP). FMRP is an mRNA binding protein that regulates dendritic protein synthesis. Research spanning the past two decades has identified metatrophic glutamate receptor 5 (mGluR5) as a synaptic target that is translationally regulated by FMRP and mGluR5-mediated translation is constitutive and unregulated. The mGluR Theory of FXS proposes that overactive signaling by group 1 mGluRs (mGluR1 and mGluR5) contributes to many of the psychiatric and neurological symptoms of FXS. The theory contends that FMRP binds to synaptic mRNAs and represses their translation. Upon mGluR activation, FMRP is inactivated or dislodged from target mRNAs, and rapid dendritic synthesis of new proteins leads to long-term depression (LTD) at locally active synapses. In the absence of mGluR activation, FMRP is translationally regulated by FMRP and mGluR5 (Westmark and Malerz, 2007). App mRNA codes for a transmembrane protein amino-iso-beta protein precursor (APP), which is processed by β- and γ-secretases to generate amyloid-beta (Aβ), the predominant protein found in the senile plaques characteristic of Alzheimer’s disease (AD) and Down syndrome. Fmr1KO mice, which lack that translational repressor FMRP, exhibit elevated levels of brain APP and Aβ, and the brains of FXS patients also appear to have elevated Aβ (Westmark et al., 2011b). Importantly, downregulation of APP and consequent reduction of Aβ can rescue many phenotypic abnormalities of Fmr1KO mice (Westmark et al., 2011b). Thus, it is our opinion that therapies directed at normalizing APP and Aβ levels will benefit FXS. Our opinion is relevant and timely as β-site APP cleaving enzyme (BACE-1) inhibitors are entering clinical trials for the treatment of FXS, which is considered an orphan disease from the standpoint of treatment development. Herein, we provide a framework for preclinical studies validating APP and Aβ pathophysiology and BACE-1 inhibitor efficacy in animal models of FXS.

THE mGluR THEORY OF FXS

“The mGluR Theory of Fragile X” proposed by Bear et al. (2004) proposes that overactive signaling by group 1 mGluRs (mGluR1 and mGluR5) contributes to many of the psychiatric and neurological symptoms of FXS. The theory contends that FMRP binds to synaptic mRNAs and represses their translation. Upon mGluR activation, FMRP is inactivated or dislodged from target mRNAs, and rapid dendritic synthesis of new proteins leads to long-term depression (LTD) at locally active synapses. In the absence of mGluR activation, FMRP is translationally regulated by FMRP and mGluR5 (Westmark and Malerz, 2007). App mRNA codes for a transmembrane protein amyloid-beta protein precursor (APP), which is processed by β- and γ-secretases to generate amyloid-beta (Aβ), the predominant protein found in the senile plaques characteristic of Alzheimer’s disease (AD) and Down syndrome. Fmr1KO mice, which lack that translational repressor FMRP, exhibit elevated levels of brain APP and Aβ, and the brains of FXS patients also appear to have elevated Aβ (Westmark et al., 2011b). Importantly, downregulation of APP and consequent reduction of Aβ can rescue many phenotypic abnormalities of Fmr1KO mice (Westmark et al., 2011b). Thus, it is our opinion that therapies directed at normalizing APP and Aβ levels will benefit FXS. Our opinion is relevant and timely as β-site APP cleaving enzyme (BACE-1) inhibitors are entering clinical trials for the treatment of FXS, which is considered an orphan disease from the standpoint of treatment development. Herein, we provide a framework for preclinical studies validating APP and Aβ pathophysiology and BACE-1 inhibitor efficacy in animal models of FXS.
zebrafish (Danio rerio) disease models (McBridge et al., 2005; Yan et al., 2005; Tucker et al., 2006; de Vrij et al., 2008; Michalon et al., 2012). In addition, numerous signaling molecules, convergent signaling pathways and other membrane receptors have been identified that contribute to the abnormal synaptic plasticity observed in FXS. Other interacting, and in some cases overlapping, theories have emerged. “The cAMP Theory of FXS” suggests that alterations in cAMP production contribute to FXS neuropathology (Kelley et al., 2007). The “The GABAAR Hypothesis” postulates that GABAAR is a potential therapeutic target because GABAergic agonists rescue behavioral symptoms of FXS (Heulems et al., 2012). Key FMRP ligands coding for “LTD proteins” have been identified and are potential therapeutic targets (Luscher and Huber, 2010).

APP AT THE FXS SYNAPSE

We identified App mRNA as a synaptic target for mGluR5/FMRP regulation (Westmark and Malter, 2007). FMRP binds to a guanine-rich region in the coding region of App mRNA and inhibits translation (Westmark and Malter, 2007; Lee et al., 2010). Stimulation with the group 1 mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG) releases FMRP from the App mRNA resulting in increased APP production. In Fmr1KO synaptoneurosomes and primary neurons, which lack FMRP, basal APP levels are elevated (Westmark and Malter, 2007; Liao et al., 2008) and do not change in response to DHPG. Consistent with these findings, Aβ levels are elevated in the brain of Fmr1KO mice, and several FXS phenotypes including mGluR-LTD can be rescued by genetically reducing APP and Aβ levels (Fmr1KO/Fpr KO mice; Westmark and Malter, 2007; Westmark et al., 2011b).

Amyloid-beta protein precursor functions in synapse and dendritic spine formation, synaptic transmission, and learning and memory (Hoe et al., 2012). Expression is developmentally regulated with maximal levels during synaptogenesis and subsequent decline when mature connections are completed. Pathological examination of brains from FXS patients shows an increased density of long and tortuous dendritic spines. Similarly, Fmr1KO mice exhibit elevated spine protrusion length compared to wild type (WT) littermates. Similarly, “too much” or “too little” APP and Aβ in Fmr1KO mice exacerbates audiogenic seizures (Westmark et al., 2010, 2013). These data support the requirement for maintenance of homeostatic levels of key synaptic proteins in the treatment of FXS and suggest that therapeutic dosages need to be tightly regulated. In fact, it is likely that a cocktail of low dosage drugs will be required to maintain synaptic homeostasis. The results of early-phase clinical trials with targeted FXS therapeutics have been reviewed (Berry-Kravis et al., 2011; Gross et al., 2012). Surprisingly, several of these drugs may be effective in FXS due to off-site activities that modulate APP, Aβ, and/or BACE-1. The other listed drugs are predicted to modulate these proteins based on their mechanism of action (GABA agonist (Sun et al., 2012), mGluR5 antagonist (Westmark and Malter, 2007), glycogen synthase kinase-3 (GSK3) inhibitor (Yu et al., 2012), neurosteroid (Chen et al., 2011), statin (Kojro et al., 2001), serotonin reuptake inhibitor (Cochet et al., 2013), or antioxidants (Hoe et al., 2013)).

Amyloid-beta protein precursor and Aβ are implicated in both negative and positive feedback loops predicted to affect synaptic homeostasis. Kienetz et al. (2003) determined that neuronal activity modulates the generation and secretion of Aβ peptides from hippocampal neurons that overexpress APP. Aβ in turn selectively depresses excitatory synaptic transmission through N-methyl-d-aspartate receptor (NMDAR) thus completing a negative feedback loop. Renner et al. (2010) showed that Aβ oligomers cause dynamic redistribution of mGluR5 to synapses and thus facilitate increased mGluR5 signaling. We demonstrated that Aβ induces dendritic APP translation in primary cultured neurons through an mGluR5-dependent pathway (Westmark et al., 2011b). Together these studies suggest a positive feedback loop whereby Aβ oligomers facilitate mGluR5 signaling leading to increased dendritic APP translation, which provides more target for amyloidogenic processing and the generation of additional Aβ (Ferreira and Klein, 2011; Westmark, 2013).

SECRETASES MODULATE APP PROCESSING

Anti-Aβ therapies and secretase inhibitors are leading strategies for reducing Aβ in AD. Aβ immunotherapy has proved very effective.
FIGURE 1 | Amyloid-beta protein precursor and Aβ are key regulators of synaptic activity. APP is processed by α-, β-, and/or γ-secretases to produce soluble N-terminal domains of APP (sAPPα and sAPPβ), Aβ, and C-terminal fragments. Aβ increases LTD, endo-TP (Koffie et al., 2011), induces calcium-dependent synaptic vesicle depletion at the presynaptic membrane (Parodi et al., 2010), leads to numerous postsynaptic surface proteins including NMDARs (Deniz and Parsons, 2013), activates mGluR5 signaling (Kley et al., 2008), induces Akt and APP expression (Bass et al., 2004; Westmark et al., 2011b) and interferes with normal NMDAR and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid receptor (AMPAR) trafficking by triggering receptor internalization (Knoch et al., 2008; Xia and Klann, 2012). Group 1 mGluRs are anchored to NMDAR via a chain of scaffolding proteins including the long isoforms of Homer, Shank, and postsynaptic density protein 95 (PSD95). Aβ induces disassembly of Shank1 and Homer1b clusters (Roselli et al., 2009). Group 1 mGluRs are upstream of two pivotal signaling pathways, PI3kinase/Akt/mTOR (mammalian target of rapamycin)/p70S6K and Ras/MAPK (mitogen-activated protein kinase)/p90S6K. Both of these pathways regulate the phosphorylation status of eIF4E, 4E-BPs, and ribosomal protein S6, which positively influence protein synthesis. Aβ and sAPP activate MAPK (Young et al., 2009; Chasseigneaux et al., 2011). Aβ both inhibits and activates mTOR signaling (Xia et al., 2008; Cassarino et al., 2011), and activates GSK3β (Szabadkai et al., 1998), a key regulator of numerous signaling pathways. APP and Aβ and the intracellular C-terminal fragments also affect synaptic homeostasis. APP anchors cytoplasmic polyadenylation element binding factor (CPEB) to membranes and promotes polyadenylation-induced translation (Cao et al., 2005). sAPPα increases de novo protein synthesis (Claasen et al., 2009), enhances LTP (Fayer et al., 2008), shifts the frequency-dependency for induction of LTD (Hould et al., 1997), and disrupts APP dimers at the plasma membrane (Sicile et al., 2009). While there is only a 17-amino acid difference between the differentially processed N-terminal fragments, sAPPα possesses synaptic and neuroprotective activities while sAPPβ can be toxic (Heng and Koo, 2011). The C-terminal fragment generated after amyloidogenic processing of APP is also neurotoxic and activates GSK3 (Ryan and Pimplikar, 2006). The 104 amino acid C-terminal fragment containing Aβ impairs LTP (Rabilloud et al., 1997). The levels of many synaptic proteins corresponding to a number of FMRP target mRNAs are constitutively elevated in the Fmr1KO mouse. A few examples that are regulated by mGluR5 are illustrated (ARC, activity-regulated cytoskeleton-associated protein), FMRP, MAP1B (microtubule-associated protein 1B), striatal-enriched protein tyrosine phosphatase (STEP), and APP (Bassell and Warren, 2008; Goebel-Grody et al., 2012). Overall, these data strongly suggest that APP and Aβ are among key LTD proteins whose overexpression during development play an important role in FXS pathogenesis. Key: major cellular processes (glutamate release, mGluR5 signaling, AMPAR endocytosis, translation, LTD, LTP, and APP processing) are boxed. Receptors (mGluR5, NMDAR, and GABABR1) are inserted into the membrane and differentially colored. Scaffolding (Homer, Caveolin, and PAK), signaling molecules (mAP, mTOR, and GSK3), and translated proteins (ARC, FMRP, PS098, and MAP1B) are oval-shaped and differentially colored. APP is colored fuchsia with the Aβ portion in light pink for amyloidogenic processing (β- and γ-secretase cleavage) and white for non-amyloidogenic processing (α-secretase cleavage). Secretases are denoted by the scissors symbols. Sharp arrowheads denote activation of a protein or process and rounded arrowheads denote inhibition of a protein or process.
in reducing soluble Aβ, amyloid plaque and soluble tau as well as associated cognitive decline in AD mouse models; however, there are safety questions (Morgan, 2011). An alternative approach is modulation of secretase activity. β- and γ-secretase inhibitors are currently in the preclinical stage of investigation for AD and could provide a means to reduce amyloidogenic processing in FXS. BACE-1 is a type I transmembrane aspartyl protease that functions as the rate limiting step in the generation of Aβ. A BACE-1 inhibitor significantly reduces plasma and brain Aβ in AD model mice (Ghosh et al., 2008, 2012; Chung et al., 2011). The potential advantage of BACE-1 inhibitors over mGluR5 antagonists and anti-Aβ immunotherapy is that the latter therapies can reduce APP and soluble APPα (sAPPα) through translational repression and immunodepletion, respectively. APP has normal physiological functions related to synapse formation so it would be advantageous to reduce Aβ while maintaining APP and sAPPα levels. We observed exacerbation of FXS phenotypes in Fmr1<sup>−/−</sup> mice treated with a high dose of anti-Aβ or genetically null for APP (Fmr1<sup>−/−</sup>/App<sup>−/−</sup> mice) suggesting that over-reduction of APP or a catabolite (presumably Aβ) is as toxic as over-expression likely due to the loss of neuroprotective sAPPα. α- and γ-secretases are additional drug targets for reducing Aβ levels. Activation of α-secretases, which cleave within the Aβ transmembrane region, would increase the production of the neuroprotective sAPPα fragment and decrease Aβ. The problem associated with the use of α- or γ-secretase drugs is that they modulate proteolytic processing of other proteins that are critical for cellular function (Vincent and Govitrapong, 2011; Wolfe, 2012). Thus, in our opinion inhibition of BACE-1 is a plausible therapeutic strategy to reduce Aβ and rescue ensuing phenotypes in Fmr1<sup>−/−</sup> mice while maintaining APP and sAPPα levels. Unfortunately, the design of BACE-1 inhibitors has proven challenging due to the large size of the catalytic pocket of the enzyme (Gravitz, 2011). As a result, currently identified BACE-1 inhibitors are largely excluded from reaching the central nervous system. BACE-1 inhibitors currently in trials, although able to cross the brain–blood barrier (BBB), display limited brain bioavailability. Therefore, approaches that affect BACE-1 expression levels rather than catalytic activity are being actively sought.

### Table 1 | Expected effects of drugs in clinical trials for FXS on APP, Aβ, and/or BACE-1.

| Drug (clinical trial sponsor) | Drug activity | Expected effect | Reference |
|--------------------------------|---------------|-----------------|-----------|
| Acamprosate (Indiana University) | GABA<sub>α</sub><sub>4</sub> agonist | ↓ Aβ endocytosis | Erickson et al. (2009) |
| AFG0566 (Novartis Pharmaceuticals) | mGlu5 antagonist | ↓ APP and Aβ | Levegna et al. (2011) |
| Donepezil (Stanford University) | Acetylcholinesterase inhibitor, | ↓ BACE-1 and Aβ | Ni et al. (2009); Sahu et al. (2012) |
| Fenobam (Neuropharm Ltd; FRAXA) | mGlu5 antagonist | ↓ APP and Aβ | Berry-Kravis et al. (2008, 2009), Matter et al. (2010) |
| Lithium (FRAXA) | GSK3 inhibitor | ↓ Aβ | Heusler et al. (2012) |
| Lovastatin (FRAXA) | Statin | ↓ Aβ and ↑ sAPPα | Kojro et al. (2001), Asai et al. (2010), Osterweil et al. (2013) |
| Memantine (Indiana University) | NMDAR antagonist | ↓ APP and Aβ | Erickson et al. (2008, 2009), Ray et al. (2010) |
| Minocycline (UC-Davis; FRAXA) | Antibiotic (tetracycline derivative) | ↓ BACE-1, ↑ sAPPα | Barcelo et al. (2010), Siopi et al. (2011) |
| Nolvasp (FRAXA) | GABA<sub>α</sub><sub>4</sub> antagonist | ↓ APP and Aβ | – |
| Paroxetine (FRAXA) | Serotonin reuptake inhibitor | ↑ ADAM10 and sAPPα | Indah Winarni et al. (2012) |
| SERTX157 (Seaside Therapeutics) | mGlu5 antagonist | ↓ APP and Aβ | – |
| STX205 (Seaside Therapeutics) | GABA<sub>α</sub><sub>4</sub>agonist | ↓ Aβ endocytosis | Berry-Kravis et al. (2012) |
| Vitamin C and E (MIBHR<sup>1</sup>) | Antioxidants | ↓ Aβ | – |

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**ER-BASED ACETYLTRANSFERASES REGULATE BACE-1 LEVELS AND ACTIVITY**

As part of our AD-related research, we discovered a novel form of post-translational regulation of membrane proteins that has a dramatic impact on BACE-1 metabolism. Specifically, nascent BACE-1 is acetylated in the lumen of the endoplasmic reticulum (ER). The acetylated intermediates are able to reach the Golgi apparatus and complete maturation whereas non-acetylated intermediates are retained in the ER/Golgi intermediate compartment (ERGIC) and degraded (Costantini et al., 2007; Jonas et al., 2008; Pehar and Puglielli, 2013). We identified two novel acetyltransferases (Ataases), Ataase1 and Ataase2, which acetylate BACE-1 and thus regulate its levels and activity (Costantini et al., 2007; Ko and Puglielli, 2009). Both Ataases are associated with ER and ERGIC membranes, have one single membrane domain, have a highly conserved catalytic domain that faces the lumen of the...
organellar (Ko and Puglielli, 2009), are expressed in neurons, and are upregulated in AD brain (Ding et al., 2012). We also identified two novel biochemical compounds, compound 9 (6-chloro-5H-benzo[a]phenoxazin-5-one) and compound 19 (2-chloro-3-(2-ethoxyanilino)-1,4-dihydropthalonaphthene-1,4-dione), that target Atase-1 and Atase-2 with high specificity and no apparent off-site effects (Ding et al., 2012). In cellular (Ding et al., 2012) and animal models of AD, these compounds dramatically reduce Aβ. Importantly, preliminary studies show that pharmacologic inhibition of Atase1 and Atase2 rescues synaptic deficits and extends the lifespan of APP-expressing mice without evident toxicity. Atase1 and Atase2 display important structural differences from Atase inhibitors in attenuating disease phenotypes. Our ultimate hypothesis driving our translation plan is that biochemistry of APP, Aβ, and BACE-1 in FXS and the efficacy of the Atase inhibitors in attenuating disease phenotypes. Our ultimate goal is to generate the necessary preclinical data for a BACE-1 inhibitor trial in FXS. Inhibition of BACE-1 with Atase inhibitors potentially offers several advantages in the treatment of amyloidogenic disorders including substrate specificity and BBB penetration.

In Step 1, we propose to assess BACE-1 knockdown in Fmr1KOCre/Fmr1KO/iBACEHET mice on established FXS phenotypes. The creation of tetracycline-inducible Cre/Fmr1flox/flox/BACE-1flox/flox mice would allow for genetic knockdown of BACE-1 at varied points in development (gestational, postnatal, and adult) prior to assessing rescue of phenotypes (seizures, electrophysiology, behavior, sleep, dendritic spine, and biomarker expression). The timing of BACE-1 knockdown with Cre technology could provide valuable data regarding the optimal subject age for therapeutic treatments. Chronic pharmacological inhibition of mGluR5, reversed established FXS phenotypes in adult Fmr1flox/flox mice (Michalon et al., 2012), and a single dose, open-label clinical trial of the mGluR5 antagonist fenobam improved prepulse inhibition in adult FXS patients (Berry-Kravis et al., 2009); however, earlier intervention may show improved efficacy. Results with the Fmr1flox/flox/BACE-1flox/flox mice could then be used as an efficacy standard for pharmacological BACE-1 interventions. Of note, BACE-1 knockdown in Fmr1flox/flox mice is expected to reduce Aβ and rescue hyperexcitability and seizures; however, these phenotypes are exacerbated in BACE-1flox/flox mice (Hu et al., 2010). Thus, we propose to reduce, not obliterate, BACE-1 activity as some Aβ is required for synaptic homeostasis.

In Step 2, we propose to study the pathophysiology of APP and Aβ in after flies. Drosophila melanogaster contain both the after and APP genes, which are closely related to the mammalian FMR1 and APP genes, and share many of the same disease-related phenotypes. Flies are a less expensive, well-established FXX model (Bushey et al., 2011; McBrade et al., 2012; Tesser and Brodie, 2012) and genetic crosses have the potential to elucidate the roles of APP and Aβ in learning, memory, sleep/wake cycles, and biomarker expression.

In Step 3, we propose to inhibit Atase1 with compound 9 in FXS mouse, fly, and human models. Compound 9 efficacy can be compared with BACE-1 knockdown mice, other BACE-1 inhibitors, and anti-Aβ therapies. In addition, the effect of compound 9 on APP processing can be assessed in peripheral blood mononuclear cells (PBMC) isolated from FXS patients. Preliminary studies from our laboratory indicate that Aβ is a potential blood-based biomarker for FXS (Westmark et al., 2011a,b); thus, it is important to understand the effects of BACE-1 inhibitors on both brain and systemic Aβ levels in FXS. Overall, these complementary but distinct approaches to study the biology of APP, Aβ, and BACE-1 in FXS and to rescue disease phenotypes in response to compound 9 could provide solid preclinical data to support testing BACE-1 inhibitors in FXS clinical trials.

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