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Fragile X and APP: A Decade in Review, A Vision for the Future.

Cara J. Westmark

Abstract

Fragile X syndrome (FXS) is a devastating developmental disability that has profound effects on cognition, behavior and seizure susceptibility. There are currently no treatments that target the underlying cause of the disorder and recent clinical trials have been unsuccessful. In 2007, seminal work demonstrated that amyloid-beta protein precursor (APP) is dysregulated in *Fmr1*^{KO} mice through a metabotropic glutamate receptor 5 ($mGluR_5$)-dependent pathway. These findings raise the hypotheses that: (1) APP and/or APP metabolites are potential therapeutic targets as well as biomarkers for FXS, and (2) $mGluR_5$ inhibitors may be beneficial in the treatment of Alzheimer's disease. Herein, advances in the field over the past decade that have reproduced and greatly expanded upon these original findings are reviewed, and required experimentation to validate APP metabolites as potential disease biomarkers as well as therapeutic targets for FXS are discussed.

Keywords

Amyloid-beta protein precursor (APP); cellular prion protein (PrP^C); fragile X mental retardation protein (FMRP); fragile X syndrome (FXS); metabotropic glutamate receptor 5 ($mGluR_5$)

1. Background

Fragile X syndrome (FXS) is a neurodevelopmental disorder clinically characterized by intellectual disability (overall IQ<70), autistic-like behaviors and seizures [1]. FXS results from a mutation in a single gene on the X chromosome, *FMR1*, that is associated with transcriptional silencing of the *FMR1* promoter and loss of expression of fragile X mental retardation protein (FMRP) [2]. FMRP is a multi-functional mRNA binding protein involved in the transport, localization and translational repression of a subset of dendritic mRNAs [3–6]. In dendrites, FMRP is predominantly found in the post-synaptic density associated with polysomes or nontranslating ribonucleoprotein (RNP) particles [7–9]. Hundreds of mRNA ligands that bind to FMRP have been identified with many having the potential to influence synapse formation and synaptic plasticity [10–12]. FMRP expression is absent or greatly reduced in FXS, and many FXS phenotypes are manifested in *Fmr1*^{KO} mice, which lack expression of FMRP [13]. *Fmr1*^{KO} mice exhibit many of the physical and behavioral characteristics of humans with FXS and are thus the most widely employed, non-human

*Corresponding Author Information: Cara J. Westmark, Assistant Professor, Department of Neurology, University of Wisconsin-Madison, Medical Sciences Center, Room 3619, 1300 University Avenue, Madison, WI USA, westmark@wisc.edu, Phone: (608) 262-9730.

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model system available for testing interventions. The leading drug target to date is the glutamate-activated, G-protein-coupled receptor metabotropic glutamate receptor 5 ($mGluR_5$), which is widely expressed in the CNS, generally postsynaptic in location and signals through FMRP [14].

In 2007, seminal findings were published demonstrating that FMRP binds to the coding region of *App* mRNA at a guanine-rich, G-quartet-like sequence and regulates amyloid-beta protein precursor (APP) synthesis through a $mGluR_5$-dependent pathway [15]. APP is processed by β- and γ-secretases to produce amyloid-beta (Aβ), the predominant peptide found in the senile plaques in Alzheimer's disease (AD). Thus, these data suggest a possible link between AD and FXS, and evoke the hypothesize that APP and Aβ can be developed as biomarkers for disease severity and drug efficacy and that drugs under study for AD can be repurposed for FXS. Over the past decade, these original findings have been independently reproduced and advanced by many laboratories (Figure 1). Herein, those findings are reviewed and a vision for future FXS-APP research is proposed.

2. FMRP Represses APP Translation Through a Guanine-Rich Element in the Coding Region of *App* mRNA

FMRP monomer binds directly to a guanine-rich region (nucleotides 699–796) in the coding region of mouse *App* mRNA and is also part of a complex that protects a *cis*-regulatory element in the 3'-untranslated region (UTR) located approximately 200 bases downstream from the stop codon (nucleotides 2318–2416) (GenBank accession number X59379) [15]. Activation of $mGluR_5$ rapidly increases translation of APP by displacing FMRP from the guanine-rich region of *App* mRNA [15]. In the absence of FMRP ($Fmr1^{KO}$), APP levels are constitutively increased [15]. Of critical importance to this topic, in 2010, FMRP was found to bind to the guanine-rich region of human *APP* mRNA [16]. It was demonstrated that the RNA binding proteins (RBP) hnRNP C and FMRP associate with the guanine-rich coding region element in human *APP* mRNA to influence APP translation competitively in opposite directions [16]. Specifically, FMRP represses translation by recruiting *APP* mRNA to processing bodies (P-bodies). Another RBP, heterogeneous ribonucleoprotein C (hnRNP C), promotes APP translation by displacing FMRP [16]. FMRP does not repress APP translation in the absence of the processing-body proteins RCK, Ago1 or Ago2 [16]. The interaction of hnRNP C with mouse *App* mRNA is significantly more abundant in lysates prepared from $Fmr1^{KO}$ than from WT brain, and associates with higher expression of APP in $Fmr1^{KO}$ mice [16]. This work confirms the findings that FMRP binds to a guanine-rich region in the coding region of *App* mRNA and that APP levels are elevated in $Fmr1^{KO}$ mice, while utilizing a human neuroblastoma cell line, which generalizes the findings between species. Both laboratories found that steady state abundance of mouse *App* mRNA in whole brain is comparable between WT and $Fmr1^{KO}$ mice indicating a translational, as opposed to posttranscriptional mRNA stability, mechanism [15,16]. Overall, the findings suggest a paradigm whereby hnRNP C promotes translation of *APP* mRNA by competing with FMRP for interaction at the guanine-rich coding region element in *APP* mRNA, thereby preventing the localization of the message by FMRP to P-bodies.

Two independent laboratories employing crosslinking immunoprecipitation (CLIP) methodologies also found that FMRP binds to *APP*/*App* mRNA. First, *App* mRNA was identified using high throughput sequencing of RNAs isolated by CLIP to identify FMRP interactions with mouse brain polyribosomal mRNAs [17]. And second, FMRP binding to the guanine-rich region of *APP* mRNA was confirmed *in vitro* using electromobility shift assays (EMSA) with recombinant FMRP and radiolabeled fragments of *APP* mRNA [18]. Three photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP)-identified FMRP target sites within *APP* (see Supplementary Figure 7 within [18]) were tested. *Site 1* in *APP* mRNA (nucleotides 888–948, NM_000484) overlaps with the guanine-rich region identified as binding to FMRP [15]. *Site 2* (nucleotides 2169–2228, coding region) and *site 3* (nucleotides 3337–3396; 3'-UTR) PAR-CLIP sites also bind to recombinant FMRP; however, binding is significantly stronger to *site 1* [18]. Please note, the nucleotide citations differ for mouse (*App*) and human (*APP*) genes. GenBank accession numbers have been provided for alignment purposes. *Site 1* of the human gene (nucleotides 888–948, NM_000484) overlaps with nucleotides 699–796 of the coding region of the mouse *App* gene (X59379).

Two additional laboratories using bioinformatics approaches provided complementary data supporting the importance of the guanine-rich coding region element in *App/APP* mRNA. First, the catRAPID method, which identifies potential interactions between protein and RNA molecules, predicted that FMRP binds to nucleotides 650–751 and 751–852, which is the experimentally validated region encompassing nucleotides 699–796 in the coding region of *App* mRNA [19]. Second, Quadparser found a putative G-quadruplex in the coding region of *APP* mRNA consistent with earlier findings [20]. The G-quadruplex is predicted to be relatively weak because the intervening loops are relatively long with four nucleotides each allowing a quadruplex with only two stacks of guanine tetrads [20]. These data corroborate the observation that FMRP monomer binds to the guanine-rich region immediately upstream from the predicted G-quadruplex and not to the G-quadruplex. In addition to the G-quadruplex in the coding region of *APP* mRNA, there is a relatively strong G-quadruplex in the 3'-UTR (739 bases downstream from the stop codon) that has no apparent role in regulating transcription or mRNA stability but negatively regulates APP levels [20,21]. It is interesting that both of the predicted G-quadruplexes occur immediately downstream of known *cis*-regulatory elements in *App* mRNA. There is an 81-nt *cis*-regulatory element in the 3'-UTR of *APP* mRNA that forms part of a 68 kD RNA-protein complex involved in TGFβ-induced stabilization of the message [22]. The last three bases (GGG) of the 81-nt *cis*-element overlap with the first three bases of the second G-quadruplex. These data suggest that the G-quadruplexes in *APP* mRNA may act as protein loading signals for upstream *cis*-regulatory elements. Overall, the bioinformatics approaches predict the experimental observation that FMRP binds to the guanine-rich coding region element in *App* mRNA.

The detailed mechanism(s) through which FMRP represses the translation of APP remain to be determined. Current findings suggest a competition between FMRP and hnRNP C for binding to the guanine-rich region in the coding region of *APP* mRNA [16]. FMRP interacts with Ago proteins and the microRNA pathway [23,24], and microRNAs have been shown to target *APP* mRNA and reduce APP expression [25]. Of significant importance to this topic,

in 2017, findings were published indicating that inhibition of the noncoding RNA (ncRNA) BC1, or specifically the BC1-FMRP association in Tg2576 AD mice, blocks aggregation of Aβ in the brain and protects against spatial learning and memory deficits [26]. In contrast, expression of exogenous BC1 in excitatory pyramidal neurons of mice induces Aβ peptides accumulation and impairs spatial learning and memory [26]. The combination of *in vitro* and *in vivo* data indicates that when BC1 associates with FMRP, FMRP does not bind to *APP* mRNA, allowing for increased translation and processing of APP resulting in increased Aβ levels and spatial learning and memory deficits [26]. Conversely, inhibition of BC1 reduces full-length APP, Aβ peptides and Aβ plaques without affecting *APP* mRNA levels [26]. In total, these findings suggest a molecular mechanism involving FMRP, hnRNP C and BC1 in the regulation of APP synthesis and are of interest in the context of FXS.

The role of BC1 in FXS has been hotly debated as there is contradictory evidence regarding if FMRP associates directly with dendritic BC1 RNA [27,28]. One set of evidence suggests that FMRP binds directly to BC1 RNA and that blocking BC1 inhibits the interaction of FMRP with its target mRNAs, which elicits the hypothesis that BC1 RNA acts as a bridge between FMRP and its target mRNAs [29,30] (*Model 1*). Other laboratories independently set out to test the BC1-FMRP model and could not document specific BC1-FMRP interactions *in vitro* or *in vivo* [31]. Their data suggest that the interactions between BC1 RNA and FMRP target mRNAs are nonspecific and support a model in which BC1 RNA and FMRP are translational repressors that operate independently (*Model 2*). Neither study tested BC1 binding to *App* mRNA. While subsequent work studying BC1 in the context of FMRP and *APP* mRNA, does not resolve the controversy [26], it does shed light on the mechanism underlying BC1 regulation of APP translation, which appears to support aspects of both previously published models. The subsequent study employed *in vitro* EMSA conditions containing physiological salt concentrations and competitor RNA and showed BC1 binding to truncated recombinant FMRP (amino acids 1–60) [26]. Nonetheless, recombinant RBPs can be notoriously “sticky” causing inherent specificity problems in EMSA. However, there is elegant *in vivo* work directly assessing BC1 effects on APP in Tg2576 mice through over- and underexpression of BC1 and *in vivo* competition assays with Tat-FMRP peptides that strongly supports a model whereby BC1 binds to FMRP, which then cannot bind to and repress *APP* mRNA, thus promoting APP synthesis [26] (*Model 3*). Thus, *Models 1* and *3* concur in supporting the binding of BC1 and FMRP. *Model 3* also indicates that FMRP binding is mutually exclusive in that it only binds to *APP* mRNA or BC1 but not both at the same time, which concurs with *Model 2* that FMRP binds directly to its mRNA targets. $BC1^{KO}Fmr1^{KO}$ mice exhibit exacerbated synaptic hyperexcitability and epileptogenesis as well as deficits in place learning supporting a sequential-independent modus operandi for BC1 and FMRP [32]. *Model 3* is congruent with *Model 2* in that BC1 and FMRP appear to act in a sequential-independent manner; however, in the case of *APP* mRNA, BC1 activates rather than represses translation. It has been hypothesized that there could be two pools of mRNA that are in competition for the translational machinery and repression of pool 1 by FMRP would allow for efficient translation of pool 2 [14]. Likewise, pools of mRNA could be differentially regulated by BC1, which in the case of *APP* mRNA, appears to bind to and compete FMRP away from *APP* mRNA leading to increased production of APP and Aβ.

Model 3 employs the Tg2576 AD mouse model, which harbors a human *APP* cDNA with the *Swedish* familial AD mutation (APP_{SWE}) but no UTR sequences [33]. Thus, the effects of over- and under-expression of BC1 RNA on APP translation in this model can be attributed to the coding region of *APP* mRNA. FMRP also protects at least one region of the 3'-UTR known to bind to other RBP including nucleolin and hnRNP C [15]. It is not known how interactions between multiple *cis*-regulatory elements, RBP, ncRNA and *APP* mRNA affect mRNA localization, stability and protein synthesis.

3. APP and Aβ Levels are Elevated in Mouse *Fmr1*^{KO} Models

Steady state levels of APP are substantially higher in *Fmr1*^{KO} synaptoneurosomes (SN) prepared from juvenile mouse cortices (postnatal day 14–17; P14–17) and in primary cultured *Fmr1*^{KO} neurons compared to WT controls [15]. This work has been partially substantiated by two other laboratories by western blot analysis, which indicates an approximately 1.7-fold increase in APP in *Fmr1*^{KO} versus WT SN prepared from P14 mouse cortices [34], as well as a significant upregulation of APP expression in whole brain lysates in *Fmr1*^{KO} at P21, P30 and P90 [35]. The last study found no difference in APP expression in whole brain lysates comparing WT and *Fmr1*^{KO} at P7 and P14 [35]. In total, three independent studies in SN or whole brain lysates prepared from postnatal mice demonstrate increased APP levels in *Fmr1*^{KO} mice albeit there were varied results at P14. These results indicate that the absence of FMRP leads to increased APP expression in the brain during a period that is critical for the stabilization of synapses.

Regarding APP levels in primary cultured *Fmr1*^{KO} neurons, SILAC (stable isotope labeling by amino acids in cell culture) experiments indicate decreased APP expression in *Fmr1*^{KO} [34]. The opposite results between western blot analysis of the synaptic fractions (1.7-fold increase) and SILAC analysis of cultured neurons (2–3.4-fold decrease) may be attributed to differences in protein stability in different assay buffers and/or to differential regulation of APP at varied developmental stages [34]. As two independent studies both prepared primary cultured neurons from embryonic day 18 mice [15,34], lower detection of APP by SILAC versus immunofluorescence could be due to a stability or solubility issue with the SILAC or to differences in local protein concentration with immunofluorescence. SILAC requires harvesting total protein from the neurons prior to digesting the protein and analysis by mass spectrometry whereas immunofluorescence involves fixing primary cultured neurons and screening APP expression in dendrites by confocal microscopy after staining with an APP-specific antibody. Of the 10 SILAC-identified proteins that underwent validation by western blot, 8 proteins exhibited good correlation between the two methods [34]. The two proteins with opposite responses were APP and α-synuclein, which are high molecular weight membrane bound proteins that would be expected to have solubility issues [34]. Alternatively, the proteins could have been diluted in the whole cell lysates or there could be differences in distribution between dendritic puncta and cell bodies. Further support implicating FMRP in the regulation of APP during embryogenesis comes from studies in mouse embryonic stem cells (mESC) where APP levels increase as FMRP is downregulated [36]. In the final analysis, the majority of the data support higher APP levels in *Fmr1*^{KO}, and there are numerous possible explanations for the discrepancy with the SILAC data.

There appears to be an age-dependent transition in APP levels and/or processing in *Fmr1*^{KO} mice. In adult animals, one study finds no difference in brain APP or Aβ levels between WT and *Fmr1*^{KO} young adult mice (3.5 months of age) in hippocampus, cortex or cerebellum by western blotting using an anti-mouse antibody against the amino-terminus of APP [37]. Another study reports increased APP at P90 in *Fmr1*^{KO} whole brain lysates and transient overexpression of sAPP α in the *Fmr1*^{KO} at P21 and P30 but not P14 and P90 [35]. Regardless of whether this variance in APP results is due to the small difference in age of the mice (3 versus 3.5 months) or utilizing brain-specific regions versus whole brain lysates, there appears to be an age-dependent shift in APP levels between postnatal and young adult *Fmr1*^{KO} mice. Two laboratories find elevated Aβ levels in older adult *Fmr1*^{KO} mice [15,35]. In addition, there is a significant decrease in Aβ in juvenile *Fmr1*^{KO} mice compared to WT controls as well as increased soluble APPalpha (sAPPα) and decreased soluble APPbeta (sAPPβ) at P21, suggesting increased α-secretase processing in juvenile *Fmr1*^{KO} mice [35]. In total, these studies suggest that dominant APP processing may change as a function of development resulting in varied ratios of APP metabolites.

4. APP and Aβ Levels are Dysregulated in Human Fragile X Models

In human studies, there is conflicting data regarding APP metabolite levels, which may vary dependent on tissue, subject age, and sample collection methods. One study finds decreased levels of $A\beta_{1–42}$ in blood plasma from full-mutation FXS adult males with no change in APP/sAPPα or $A\beta_{1–40}$ levels [38]. Due to a limited number of FXS autopsy brain samples available for analysis, there were only trends for decreased APP/sAPPβ and increased $A\beta_{1–40}$ in FXS brain (neocortex and hippocampus). Another study finds increased levels of APP and ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10; aka α-secretase) in primary fibroblasts from adolescent and adult FXS patients and in frontal cortex of adult FXS [35]. A third study finds elevated levels of sAPP, sAPPα, sAPPβ, $A\beta_{1–40}$ and $A\beta_{1–42}$ in pediatric FXS plasma as well as elevated sAPP, sAPPα and $A\beta_{1–40}$ in FXS brain [39,40]. Interestingly, acamprosate treatment significantly reduced sAPP and sAPPα in pediatric subjects with FXS-associated autism spectrum disorder (ASD) [41]. There are potentially confounding issues with these studies. For example, blood levels of APP metabolites may not correlate with brain levels, or inversely correlate if the brain acts as sink for Aβ. Also, the anticoagulant used to collect blood can have large effects on APP metabolites, which may explain the varied results in Aβ levels in plasma (lower Aβ levels in blood collected in lithium heparin versus higher Aβ levels with EDTA) [42]. In addition, subject age varied between studies (adult versus pediatric), and similar to the *Fmr1*^{KO} mouse studies, APP processing may change as a function of age. In total, these findings support a role for dysregulated APP production and processing in FXS and indicate that the APP metabolites may be viable therapeutic targets and/or biomarkers if consistent testing conditions can be determined.

5. FMRP Expression Varies in AD Models

The flipside of APP metabolite expression in FXS models is FMRP expression in AD models. FMRP levels decrease with age in mice [43,44], supporting the hypothesis that loss of this translational regulator could contribute to increased APP synthesis and Aβ processing

with aging and thus the development of AD. There are some complicated data from the AD field in regard to this hypothesis, which is summarized (Table 1). One laboratory finds no difference in FMRP expression in AD model mice (APP_{SWE}/PS1ΔE9; 2- and 20-months old) in cerebellum or cortex compared to littermate controls albeit there was a non-significant increase in FMRP in cerebellum in the 2-month old AD mice [45]. In addition, FMRP expression is similar in frontal cortex and cerebellum samples from sporadic AD and control patient autopsy samples [45]. A second laboratory finds reduced FMRP in Tg2576 compared to WT mice (1- and 3-months old) [46]. FMRP levels are equivalent in 6-month old mice; however, FMRP levels drop dramatically with age resulting in low levels in both WT and Tg2576 [46]. This group provides complementary evidence that hnRNP C expression is elevated in 1- and 3- month old Tg2576 but not 6-month old mice compared to WT controls [46]. Furthermore, FMRP is decreased and hnRNP C increased in hippocampal SN prepared from human sporadic AD compared to healthy donors [46]. A third group finds that FMRP expression is increased in APP_{SWE}/PS1ΔE9 mice (12-months old) [47]. They propose that increased FMRP levels are a compensatory mechanism to reduce ectopic/transgenic translation of APP although that compensatory mechanism appears ineffective [47].

There are some caveats associated with these studies that could explain the different outcomes. For example, different strains and ages of AD mice were employed. Examining the entirety of the data, FMRP levels are reduced in AD mice at 1- and 3-months of age (Tg2576), equivalent at 2- (APP_{SWE}/PS1ΔE9) and 6-months of age (Tg2576), elevated at 12-months of age (APP_{SWE}/PS1ΔE9), and equivalent at 20-months of age (APP_{SWE}/PS1ΔE9) compared to WT controls suggesting a bell-shaped, age-dependent expression pattern of FMRP in the context of greatly exacerbated APP and Aβ production in AD mice with a shift in the curve dependent on the AD mouse model. The double transgenic AD mice (APP_{SWE}/PS1ΔE9) begin to develop Aβ deposits by 6 months of age compared to 11–13 months in the Tg2576 [33,48]. In the APP_{SWE}/PS1ΔE9, there is a progressive increase in plaque number up to at least 12-months of age [49]. Overall, the data support a model in which FMRP production increases with accumulating APP and Aβ followed by reduced levels upon Aβ sequestration into plaques. The early decrease in FMRP in the 1- to 3-month of age period in the Tg2576 could indicate that the compensatory mechanism does not kick in until after a threshold level of APP or metabolites is produced. These results have important implications for therapeutic timing in the treatment of AD. Regarding the varied results assessing FMRP levels in human sporadic AD tissue, the studies analyzed different brain regions (frontal cortex and cerebellum [45] versus hippocampus [46]).

Interestingly, there is reduced FMRP expression in cortex and cerebellum tissue of two of three patients with fragile X-associated tremor/ataxia syndrome (FXTAS) compared to control and AD brains, with a corresponding increase in APP in cerebellar but not cortical samples [45]. Alzheimer's disease-type pathologies (plaques and tangles) have been reported in elderly female fragile X premutation carriers [50]. *APP* is a deregulated gene in FXTAS and the corresponding mouse model, where *APP/App* mRNA is upregulated 1.87-fold in peripheral blood of FXS premutation male carriers as well as 1.38-fold in the prefrontal cortex and 1.18-fold in the brainstem of FXTAS mice [51]. These data deserve further evaluation in a larger population.

6. APP Translation is Regulated through FMRP and $mGluR_5$

Stimulation of cortical SN or primary neuronal cells with the mGluR agonist (*S*)-3,5-diydroxyphenylglycine (DHPG) increases APP levels in wild type (WT) but not $Fmr1^{KO}$ samples [15]. The increase in APP in SN can be blocked with the translational inhibitor anisomycin or the $mGluR_5$ antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) [15]. *App* mRNA co-immunoprecipitates with FMRP in resting SN but the interaction is lost after DHPG treatment [15]. *App* mRNA levels remain constant irrespective of genotype or treatment [15]. And, treatment of primary neuronal cells with $mGluR_5$ antagonists reduces steady state levels of APP [15,52]. In combination, these data demonstrate that the production of APP is regulated through a FMRP- and $mGluR_5$-dependent signaling pathway, that mGluR-dependent APP synthesis is already at maximal capacity in the $Fmr1^{KO}$ and cannot be further stimulated, and that APP translation is independent of mRNA decay. The finding that *App* mRNA levels are not affected by FMRP dosage has been replicated by two laboratories [35,36]. In addition, evidence is provided that APPα affects the overall translation of APP, genetic reduction of *App* in $Fmr1^{KO}$ mice returns synaptic *de novo* protein synthesis to WT levels, sAPPα increases *de novo* protein synthesis in primary cortical neurons, genetic reduction of ADAM10 lowers *de novo* protein synthesis in $Fmr1^{KO}$ SN, and SN prepared from APP^{KO} mice exhibit reduced protein synthesis [35]. These data strongly support a role for sAPPα in translational activation. sAPPα acts though $mGluR_5$ as MPEP blocks the sAPPα-mediated increase in phosphorylated extracellular signal-regulated kinase 1/2 ($ERK_{1/2}$). Overall, these findings implicate $mGluR_5$, FMRP and sAPPα in a feedback loop that regulates protein synthesis.

Verification of an $mGluR_5$-dependent signaling pathway that regulates APP and Aβ comes from the AD field. Aβ levels were quantitated in AD mice after chronic dosing with the $mGluR_5$ inhibitor fenobam [53]. The drug was incorporated into the feed at 0.2g fenobam per kg feed for an anticipated dose of 24–30 mg drug/kg body weight/day [53]. Drug delivery via feed is advantageous because daily oral gavage or intraperitoneal injections can be stressful to the animals. Phase I dose escalation trials show safety and a lack of cognitive dysfunction in humans receiving up to 8–9 mg/kg/day fenobam for 4 weeks albeit there were psychostimulant side effects [54–57]. The mouse dose was 3-fold higher than that safely tested in humans, but far less than that safely tested in rats [58]. As there are no reports of toxicity with the drug, an err on the side of over-dosing facilitates determining if inhibition of $mGluR_5$ affects Aβ levels or behavior. Two AD models, Tg2576 and R1.40 mice, which both overexpress the human *APP* gene with the familial Swedish mutation (APP_{SWE}, K670N/M671L) were employed. There was a large decrease in mouse Aβ levels in the Tg2576 in response to fenobam with no change in transgenic human Aβ levels [53]. In the R1.40 mice, there was a small, statistically significant decrease in Aβ [53]. The inability of fenobam to reduce human Aβ in the Tg2576 is likely due to the nature of the $hAPP_{SWE}$ transgene. In the Tg2576, only the coding region of $hAPP_{695}$ is inserted into the mouse genome (no 3'-UTR sequences) whereas the R1.40 transgene contains 250 kB of flanking sequence. FMRP binds to the 3'-UTR of *App* mRNA [15]. It is not known at this time if FMRP binds directly to the 3'-UTR or as part of a RNP complex, but it appears that regions outside of the coding region are vital for FMRP regulation of APP synthesis. The mouse

App gene in the Tg2576 and the human *APP* transgene in the R1.40 both contain coding and noncoding FMRP-dependent *cis*-regulatory elements. No adverse side effects were observed in response to chronic dosing nor any premature deaths [53]. In the Tg2576, there is a 40% death rate by 60 days of age [59]. Thus, chronic treatment with the $mGluR_5$ inhibitor fenobam reduces mouse Aβ in Tg2576 mice and human Aβ in R1.40 mice and improves survival in the Tg2576 [53].

The lack of 3'-UTR *cis*-regulatory elements in *App* transgenes may explain other unexpected findings. First, it was hypothesized that the absence of FMRP in FRAXAD mice (cross of Tg2576 and $Fmr1^{KO}$ that overexpresses both human and mouse APP in the absence of FMRP) would result in increased synthesis of APP and generation of Aβ, which would accumulate with aging [37]. No significant differences were observed in APP or Aβ levels in young adult Tg2576 and FRAXAD mice (2-months old) albeit there was an increase in APP in aged FRAXAD mice (16–18 months old) suggesting differential regulation of the endogenous murine versus transgenic human *App/APP* genes [37]. Immunofluorescence analysis of cultured Tg2576 neurons reveals that APP_{SWE} is predominantly in the soma rather than the dendrites [37]. Thus, FMRP binding to the 3'-UTR of the *APP* transgene may be required for dendritic targeting. Second, increased expression of APP would be expected in response to siRNA knockdown of FMRP in Neuro-2a cells stably expressing wild type (WT) human APP_{695} [60]. Surprisingly, FMRP siRNA does not cause increased APP levels in the control lanes (see Figure 4 within [60]). Information is not available regarding if the APP_{695} construct used contained *App* 3'-UTR *cis*-regulatory elements [60]. Third, $mGluR_5$ knockout in APP_{SWE}/PS1ΔE9 AD mice reduces spatial learning deficits, Aβ oligomer formation, Aβ plaque number, mTOR phosphorylation, and FMRP expression [47]. This suggests that a positive feedback loop involving $mGluR_5$ increases Aβ formation and AD pathology in APP_{SWE}/PS1ΔE9 mice. However, the genetic deletion of $mGluR_5$ had no apparent effect on the transgenic expression of APP in APP_{SWE}/PS1ΔE9 suggesting that Aβ levels were altered through increased cleavage and not overall synthesis. These data support the hypothesis that *APP* flanking sequences are required for $mGluR_5$/FMRP-mediated regulation of APP synthesis.

The genetic knockdown study complemented findings that blockade of $mGluR_5$ with 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl) ethynyl) pyridine (CTEP) improves memory and cognitive function in APP_{SWE}/PS1ΔE9 mice while reducing Aβ oligomers and plaques [61]. Chronic administration of CTEP is required to rescue memory deficits in novel object recognition and the Morris water maze [61]. Aβ oligomer concentrations and plaque formation are also reduced in the 3xTg-AD mice [61]. In addition, selective blockade of $mGluR_5$ is neuroprotective in cortical cultures challenged with toxic concentrations of Aβ [62]. Thus, $mGluR_5$ is a viable drug target to modulate Aβ levels and ensuing phenotypes.

7. An $mGluR_5$ Signaling Nexus Includes APP, Aβ, PrP^C and FMRP

The feedback regulation between $mGluR_5$ and APP/Aβ is complex (Figure 2). While $mGluR_5$ activation increases APP production, APP metabolites also appear to influence $mGluR_5$ expression and localization. $mGluR_5$ transcript and protein levels increase

significantly in cells treated with Aβ [63–67]. Reactive astrocytes that surround Aβ plaques in AD mice and patients overexpress $mGluR_5$ [64,65,68]. Although there is an age-related decrease in $mGluR_5$ in WT mice, the decrease is less apparent in Tg-Arc_{SWE} AD mice resulting in a significant increase in $mGluR_5$ expression in 16-month old Tg-Arc_{SWE} mice compared to WT [69]. And, cell surface expression of $mGluR_5$ increases by 4.4-fold in APP_{SWE}/PS1ΔE9 mice compared to WT without a change in total cellular expression of $mGluR_5$ [47]. These data fit with an earlier finding that Aβ oligomers bind to and aggregate on neuronal and astrocytic plasma membranes and cause redistribution of $mGluR_5$ to synapses resulting in clusters that partially co-localize with Aβ oligomers [68,70]. In contrast, there are reported tissue and model-specific effects. For example, $mGluR_5$ protein expression increases in astrocytes from the hippocampus but not the entorhinal cortex and selectively in non-transgenic mice but not 3XTg-AD mice [71]. In hippocampus, $mGluR_5$ mRNA expression levels decrease in 9-month old but not 6-month old APP_{SWE}/PS1ΔE9 mice compared to controls, but the decrease does not result in altered $mGluR_5$ hippocampal protein levels [72]. In cortex, there is no significant difference in $mGluR_5$ mRNA or protein expression in APP_{SWE}/PS1ΔE9 versus control mice. And, $mGluR_5$ density is lower in the limbic system in 5xFAD mice [73]. In human subjects, $mGluR_5$ is expressed by 40% of striatal neurons in young individuals with significant intensity variations among neurons, which increases to 80% and 92% of striatal neurons in elderly individuals and AD patients, respectively [74]. Overall, the synergy between $mGluR_5$ and APP/Aβ is complex and may vary dependent on tissue and disease model.

The complexity of $mGluR_5$/Aβ signaling is increased by involvement of the cellular prion protein (PrP^C). $mGluR_5$ does not bind directly to Aβ, but physically associates with PrP^C, and this complex plays important roles in modulating Aβ binding and neuronal activity [75,76]. The Aβ/PrP^C/$mGluR_5$ signaling complex allows PrP^C to associate with the intracellular protein mediators such as Homer1b/c, calcium/calmodulin-dependent protein kinase II (CaMKII) and protein tyrosine kinase 2 beta (Pyk2) to affect synaptic plasticity [77–82]. Coupling of PrP^C to these intracellular proteins is modified by soluble Aβ oligomers [79]. Aβ oligomer treatment dissociates Homer1b/c and Pyk2 from the PrP^C complex while enhancing the association between PrP^C and CamKII as well as between PrP^C and $mGluR_5$. The Aβ/PrP^C/$mGluR_5$ complex also interacts with Fyn kinase, which is a tyrosine-specific phosphotransferase in the Src family of non-receptor tyrosine protein kinases [75]. A screen of 61-transmembrane postsynaptic density-enriched proteins in HEK293T cells identified $mGluR_5$ as the only candidate to mediate Aβ-induced Fyn phosphorylation in a PrP^C-dependent manner [75].

Altered Aβ/PrP^C/$mGluR_5$ complex interactions affect downstream signaling. For example, a peptide mimicking the binding site of laminin onto PrP^C (Ln-γ1) binds to PrP^C and induces intracellular Ca^{2+} increase in neurons via the PrP^C-$mGluR_5$ complex [76]. Ln-γ1 promotes internalization of PrP^C and $mGluR_5$ (decreased surface expression) and transiently decreases Aβ binding to neurons [76]. Aβ binding to the neuronal surface is also decreased in $mGluR^{KO}_5$ [70,76]. Amino acids 91–153 of PrP^C mediate its interaction with $mGluR_5$ [83]. Agonists of $mGluR_5$ or synthetic Aβ increase the $mGluR_5$-PrP^C interaction and $mGluR_5$ antagonists suppress the association. The $mGluR_5$-PrP^C interaction is enhanced dramatically in the brains of familial AD transgenic mice, but expression and activity remain to be

determined in FXS models. These data suggest that alterations in the PrP^C/$mGluR_5$/Aβ complex control downstream $mGluR_5$ signaling. Also in support of this hypothesis, (1) cortical exposure of neuronal cultures to Aβ oligomers upregulated mGluR and PrP^C α-cleavage [72]; (2) Aβ-PrP^C generates $mGluR_5$-mediated increases of intracellular calcium [75]; (3) Aβ prevents alterations in protein-protein interactions induced by group 1 mGluR activation [84]; (4) repeat treatment with the $mGluR_5$ antagonist basimglurant or an antibody that prevents Aβ oligomer binding to PrP^C causes a strong but transient reversal of the long-term potentiation (LTP) deficit in 5-month old transgenic AD rats that express human APP_{751} with the Swedish and Indiana mutations [85]; and (5) binding of BMS-984923, a potent $mGluR_5$ silent allosteric modulator, does not change glutamate signaling but strongly reduces $mGluR_5$ interaction with PrP^C bound to Aβ, which prevents Aβ-induced signal transduction in brain slices and in APP_{SWE}/PS1ΔE9 mice resulting in rescue of memory deficits and synaptic depletion [86].

Aβ affects calcium responses, long-term depression (LTD), and learning & memory through $mGluR_5$- dependent pathways. There appear to be two separate Aβ-dependent signaling cascades that affect calcium mobilization [84]. One cascade is dependent on extracellular Ca^{2+} and Fyn kinase activation and the other is dependent on the release of Ca^{2+} from intracellular stores. Aβ increases intracellular calcium mobilization in a time and concentration-dependent fashion, which is associated with a significant increase in astrocytic $mGluR_5$ mRNA and protein expression, and can be blocked by MPEP [64]. Aβ, given exogenously or by altering ADAM10 /synapse-associated protein 97 (SAP97) interactions, induces Ca^{2+} transients in astrocytes and alters Ca^{2+} release from neuronal stores through an $mGluR_5$-mediated pathway [64]. Aβ potentiates DHPG-induced Ca^{2+} responses in a cell-specific manner where DHPG significantly increases the response in Aβ-treated hippocampal but not entorhinal cortex astrocytes [71]. And, a $mGluR_5$ antagonist rescues Ca^{2+} signaling impairment in APP knockin (KI) neurons [87]. To examine the effects of Aβ on mGluR-LTD, DHPG was applied to hippocampal slices pre-treated with $Aβ_{1–42}$. While no significant difference in mGluR-LTD between slices treated with vehicle and Aβ were found, mGluRLTD was blocked in aged $APPS_{WE}$/PS1ΔE9 mice (12–15 months old) [88]. Antagonists of $mGluR_5$ prevent Aβ-induced dendritic spine loss and learning and memory deficits in 9-month old APP_{SWE}/PS1ΔE9 mice and 8–9-month old 3XTg-AD mice [75]. Overall, these data suggest that PrP^C couples Aβ with $mGluR_5$ allowing Aβ to stimulate $mGluR_5$ activities, but that Aβ can trap the PrP^C-$mGluR_5$ complex in a state that does not allow glutamate-induced regulation of the complex.

There are likely multiple feedback loops facilitating $mGluR_5$ and APP/Aβ effects. The addition of $Aβ_{1–42}$ oligomers to primary cortical neurons induces a transient increase in α-secretase activity and secreted sAPPα [89]. Preventing the generation of sAPPα with siRNAs increases $Aβ_{1–42}$ oligomer-induced cell death [89]. These data suggest that neurons respond to stress by generating sAPPα for survival. Additional feedback loops may act at the transcriptional level. Aβ binds to promoter regions of the *APP* and *BACE1* genes and may function as a transcription factor to regulate its own production and/or processing [90].

Additional evidence supporting synergy between APP and $mGluR_5$ comes from other neurological disorders. Subjects with autism exhibit altered levels of APP, FMRP and

$mGluR_5$ dependent on age and brain region. There is a significant increase in APP (120 and 88 kDA proteins) in the superior frontal cortex (Brodmann Area 9, BA9) of children with autism versus healthy controls [91], which correlates with increased levels of $mGluR_5$ [92]. This data supports the theory that elevated $mGluR_5$ signaling increases APP synthesis. Conversely, there is no change in APP (120 kDa) expression in the cerebral vermis of children with autism although $mGluR_5$ is also elevated in this brain region [91,93]. Of note, APP expression is much lower in the vermis compared to the BA9 and the antibody epitope of APP was not identified. Thus, APP expression and processing may vary with brain region. Adults with autism have significantly decreased APP (120 kDa) in vermis compared to healthy controls [91,93]. FMRP levels do not differ between control and autism BA9 child samples while there is reduced FMRP in the vermis and BA9 of adults with autism [92,93]. These data appear to contradict the model that $mGluR_5$ activation increases APP production through a FMRP-dependent pathway. However, FMRP activity is modulated by posttranslational modification status. Unphosphorylated FMRP associates with actively translating polyribosomes and a fraction of phosphorylated FMRP associates with stalled ribosomes [94]. Group 1 mGluR-induced dephosphorylation of FMRP facilitates its ubiquitination and degradation [95]. The ratios of phosphorylated serine-499 (S499)-FMRP to neuronal specific enolase (NSE) are reduced in the vermis of adults and children with autism and in BA9 of adults with autism but not in the BA9 of children with autism [96]. These data indicate a positive correlation between FMRP phosphorylation status and APP levels in autism.

In addition to autism, other neurological disorders provide complementary evidence regarding dysregulated expression of $mGluR_5$, FMRP and/or APP in brain. Patients with Down syndrome (DS) are trisomic for chromosome 21, which carries the *APP* gene. $mGluR_5$ is upregulated in astrocytes of DS brain [97,98]. FMRP is decreased in major psychiatric disorders [99], which is accompanied by altered expression of FMRP-regulated proteins [100]. Regarding APP, levels are reduced in the BA9 of patients with schizophrenia or bipolar disorder, but there are no group differences in the lateral cerebella. Interestingly, subcellular localization studies of BA9 homogenates indicate trends for reduced APP with a significant reduction in FMRP and phosphorylated FMRP in whole homogenates; elevated APP with trends for increased FMRP and phosphorylated FMRP in nuclear fractions; and reduced APP with trends for increased FMRP and phosphorylated FMRP in the rough endoplasmic reticulum (rER) [101]. Thus, in the ribosome-rich rER, there is a correlation between elevated FMRP and reduced expression of APP. $mGluR_5$ density and expression are increased in the prefrontal cortex of patients or carriers of FXS compared to healthy controls [102]. Data is not available regarding APP levels. Overall, substantial evidence suggests that dysregulated $mGluR_5$/FMRP signaling modulates APP levels in multiple neurological disorders. Of note, total FMRP levels are drastically lower in adults than in children (see Figure 2 control lanes within [93]). Much remains to be learned regarding the priority of the $mGluR_5$/FMRP/APP signaling pathway as a function of cell type and compartment, brain region, age and disease status.

Much also remains to be learned regarding the role of FMRP in $mGluR_5$/APP signaling and AD. Transcriptome analysis of brains of 2 AD mouse strains and postmortem gene expression profiles from individuals diagnosed with late onset AD (LOAD) converge on

FMR1 [103]. The mouse lines under study are APP^{E693Q} (Dutch mutation) and APP_{SWE}/PS1ΔE9. The APP^{E693Q} mice accumulate Aβ oligomers and behavioral impairment but do not develop parenchymal fibrillary amyloid deposits [103]. The APP_{SWE}/PS1ΔE9 mice accumulate fibrillar Aβ and plaques accompanied by neuritic dystrophy and behavioral impairment [103]. The LOAD tissue included profiles for 6 brain regions across 34 individuals diagnosed with LOAD along with 14 age-matched non-demented controls [103]. The *FMR1* gene is differentially spliced in fibrillogenic APP_{SWE}/PS1ΔE9, oligomerogenic APP^{E693Q} dentate gyrus, and multiple LOAD brain regions [103]. Of note, *FMR1* is differentially spliced in the frontal pole and superior temporal gyrus in LOAD [103]. There was no evidence of altered cortical or cerebellar expression of FMRP in APP_{SWE}/PS1ΔE9 nor in the frontal cortex or cerebellum of human AD postmortem samples [45]. Likewise, there was not differential expression of *FMR1*, but rather overexpression of *FMR1* exon 5 suggesting a role for alternate splicing of *FMR1* in mediating AD pathology [103]. Early work in the field indicated that the *FMR1* gene is alternatively spliced although the first splice site commenced at amino acid 282 (nucleotides 844–868) [104]. Interestingly, exon 5 of the *FMR1* gene codes for amino acids 90–139 of the amino-terminal domain of FMRP (GenBank accession number L29074.1, nucleotides 30441–30589), which overlaps with the nuclear localization signal (NLS) (amino acids 115–150) [105] and occurs upstream of the BC1 binding region (amino acids 180–217) [30,106]. A rare genetic mutation in exon 5 (arginine to glutamine at amino acid 138, R138Q) has been reported to cause FXS in 2 patients [107]. In *Drosophila*, alternative splicing of the *dfmr1* gene produces 2 isoforms that have different roles in mediating neural development and behavior [108]. The isoform lacking the glutamine/asparagine (Q/N)-rich protein interaction domain in the C-terminus of dFMRP results in defects for a subset of neural development phenotypes associated with the *dfmr1* null allele [108]. Thus, alternative splicing as well phosphorylation, ubiquitination and degradation mechanisms likely plays a role in FMRP-mediated regulation of APP. Overall, APP, FMRP and $mGluR_5$ are linked at the molecular level in activity-dependent relationships [109]

8. Functional Consequences of Altered APP Metabolite Levels in Fragile X Models

There is significant premature mortality in Tg2576 mice that overexpress human APP and Aβ (97% survival at 30 days of age; 60% at 60 days), which occurs earlier in FRAXAD mice that overexpress human APP_{SWE} in an $Fmr1^{KO}$ background (77% survival at 30 days of age; 60% at 60 days), compared to WT and $Fmr1^{KO}$ controls (100% survival at 60 days) [59]. Interestingly, an early mortality phenotype is also observed in APP_{SWE}/PS1ΔE9 mice, which exhibit a 70% survival rate at 100 days, 40% at 200 days, and 20% at 300 days of age [110]. Full genetic deletion of $mGluR_5$ or PrP^C significantly improves survival in APP_{SWE}/PS1ΔE9 to near WT levels [110].Removal of a single *Grm5* ($mGluR_5$) or *Prnp* (PrP^C) allele did not benefit survival [110]. Genetic removal of one allele of both *Grm5* and *Prnp* improves survival midway between WT and APPswe/PS1ΔE9 mice [110]. These data strongly suggest that overexpression of Aβ contributes to early mortality in AD mice and is dependent on the interaction of PrP^C and $mGluR_5$.

Early mortality may be due to lower seizure threshold. Juvenile Tg2576, FRAXAD and Ts65Dn (Down syndrome model that expresses 3 copies of murine *App*) mice (P21) are highly susceptible to audiogenic-induced seizures (AGS), which are attenuated with $mGluR_5$ antagonists or by passive immunization with anti-Aβ antibody in Tg2576 [111]. Adult Tg2576 and FRAXAD mice are highly susceptible to pentylenetetrazole-induced seizures, which are attenuated with MPEP [37,59]. Thus, seizures are associated with increased APP/Aβ levels and are reduced by inhibition of $mGluR_5$. Of note, AGS are exacerbated in both $Fmr1^{KO}/APP^{KO}$ and $BC1^{KO}Fmr1^{KO}$ mice [32,112]. At first, it seems contradictory that seizures would be exacerbated in these models as one would predict decreased expression of APP and Aβ and rescue of phenotypes; however, the data strongly suggest that both over- and underexpression of APP and BC1 increases hyperexcitability.

Restoring homeostatic APP levels in $Fmr1^{KO}$ mice rescues many FXS phenotypes including AGS, marble burying, open field, the ratio of mature to immature dendritic spines, and mGluR-LTD in $Fmr1^{KO}$ mice in response to knockdown of a single *App* allele [38]. An alternative approach, knockdown of APP in $Fmr1^{KO}$ neurons with a lentiviral vector carrying a short hairpin RNA directed specifically against *App* mRNA, reduces APP and spine density [35]. Exogenous sAPPα increases the number of immature spines while modulation of ADAM10 activity re-establishes physiological sAPPα levels and ultimately ameliorates FXS molecular, synaptic, and behavioral deficits [35]. $Fmr1^{KO}/APP^{HET}$ brain slices exhibit complete rescue of UP states in a neocortical hyperexcitability model and reduced duration of ictal discharges in a CA3 hippocampal model [113]. Thus, APP plays a pivotal role in maintaining an appropriate balance of excitation and inhibition in neural circuits. Based on these data, a model was proposed whereby $mGluR_5$ inhibitors act as a circuit breaker, FMRP as an automatic transfer switch and APP as a rheostat in a circuit that controls hyperexcitability where: (1) excess levels of APP in the $Fmr1^{KO}$ appear to cause a short circuit through overload of the APP rheostat resulting in hyperexcitability, (2) complete loss of APP bypasses the APP rheostat, and (3) the APP rheostat provides a graded response to $mGluR_5$ activation through feedback loops involving amyloidogenic and non-amyloidogenic secretase processing [113].

In support of a role for APP in hyperexcitability, 3xTg-AD mice, which harbor mutated APP, tau and presenilin (PS1) genes, were tested prior to Aβ plaque deposition, neurofibrillary pathology and cognitive impairment (3-weeks old). Results indicated that 3xTg-AD mice exhibit AGS that can be attenuated by passive immunization with anti-human APP/Aβ antibody (6E10) or blockade of $mGluR_5$ with MPEP [114]. These data confirm similar studies performed in a different AD mouse model (Tg2576) using an alternate APP/Aβ antibody (sc-28365LS) and $mGluR_5$ inhibitors (MPEP, fenobam) [111]. It is interesting to note that Tg2576 and 3xTg-AD mice exhibit a strong AGS phenotype, but J20 and R1.40 AD mice do not [111,113,114]. All of these strains overexpress human APP and Aβ; however, the J20 and R1.40 transgenes contain flanking sequences in addition to the coding sequence, which has been previously discussed in terms of the role of FMRP in regulating APP synthesis.

In addition to seizures, measuring ictal-like activity in the CA3 region of the hippocampus in 3xTg-AD mice demonstrates that epileptiform discharge duration positively correlates with

intraneuronal human APP/Aβ expression in the CA3 region, which can be suppressed with 6E10 or MPEP [114]. Specifically, in contrast to saline injected 3xTg-AD mice, none of the slices from 6E10-treated 3xTg-AD mice exhibit prolonged epileptiform discharges 60 min after bicuculline application [114]. By 90 min after bicuculline application, 34% of slices from 6E10-treated 3xTg-AD mice develop prolonged epileptiform discharges [114]. Thus, the overall incidence of bicuculline-induced prolonged epileptiform discharges is significantly reduced by passive immunization with 6E10 [114], And in slices that develop prolonged epileptiform discharges, passive immunization with 6E10 significantly increases the latency to the occurrence of these discharges [114]. Prolonged epileptiform discharges are suppressed after MPEP treatment [114]. These data strongly support roles for APP and $mGluR_5$ in network hyperexcitability and suggest that hyperexcitability is independent of plaque load. Likewise, epileptiform activity, as measured by electroencephalography (EEG) in AD mice, exhibits abnormal EEG discharges independent of plaque load [115].

The dysregulated expression of APP and metabolites in multiple FXS models elicits the hypothesis that drugs under development for AD could be repurposed for FXS [116]. In support of that hypothesis, inhibition of beta-secretase 1 (aka beta-site APP cleaving enzyme 1; BACE1) reduces AGS in juvenile *Fmr1*^{KO} mice and rescues altered morphology of *shFmr1* mouse neural progenitor cells [36,113].

9. Moving Forward

Substantial evidence has been reviewed supporting pivotal roles of APP and metabolites in FXS [117,118]. Thus, evolving AD therapies directed at modulating APP synthesis and/or processing may be applicable to FXS, and APP and metabolites may be viable blood-based biomarkers to test therapeutic efficacy and disease severity. A critical question remains regarding what preclinical studies need to be performed to further validate APP as a drug target and biomarker for FXS and help propel this target into clinical trials. Several areas of research that would increase experimental rigor and further validate the role of APP in FXS include:

1. *Study the expression and roles of APP metabolites in the brain as a function of development in FXS models*. Constitutive knockdown of APP levels in *Fmr1*^{KO} mice rescues seizures, behavior, dendritic spine, mGluR-LTD and hyperexcitability phenotypes [35,38,113]. APP metabolites are also associated with the kinetics of neurogenesis [36]. While APP expression remains stable during the differentiation of human induced pluripotent stem cells (iPSC) towards cortical neurons, APP processing changes [119]. Specifically, secretion of sAPPα is detected from day 10 of differentiation and peaks on day 75; sAPPβ is detected from day 45 and remains stable from day 75; Aβx-38 is detected from day 45 and increases thereafter; and Aβx-40 and Aβx-42 are detected at low levels from day 10 but increase significantly between days 45 and 75 [119]. Thus, there is a shift in APP processing during cortical neuronal differentiation. It will be important to determine the expression level and function of APP metabolites and secretases throughout development in FXS. This could be accomplished through conditional knockdown of *App* in *Fmr1*^{KO} flies, mice, rats

and cells as well as in FXS iPSC lines. Data to date suggest that APP processing changes as a function of age; thus; conditional expression studies will identify therapeutic windows to target specific APP metabolites.

2. *Further elucidate the protein-RNA interactions that regulate APP synthesis and their function*. Alternate models have been proposed regarding how RBP affect translation: (a) direct ribosomal recruitment, (b) inhibition of repressor binding, (c) long-range structural remodeling to remove inhibitory features, (d) biogenesis of a translation-competent mRNP, and (e) the influence of mRNA localization [120]. Binding activity and associated functions for the RBP FMRP and hnRNP C in relation to *APP* mRNA have been identified to date [15,16], but it is probable that the complexity of the protein complexes that assemble on *APP* mRNA to regulate translation resembles the more multifactorial protein-nucleic acid interactions found in transcription complexes. Much remains to be learned regarding the identity, location and function of RBP that regulate *APP* mRNA synthesis. FMRP is a known binding partner for nucleolin and YB1 [121,122]. Nucleolin, hnRNPC, RCK/p54, PAI-RBP1, autoantigen La, EF1α and YB1 bind to *cis*-regulatory elements in the 3'-UTR of *App* mRNA [123,124]. Iron regulatory protein 1 (IRP1) binds to an iron response element (IRE) in the 5'-UTR of *APP* mRNA [125]. These protein/RNA interactions could bring multiple cis-regulatory elements in *APP* mRNA into close proximity to regulate APP synthesis.

3. *Determine the role of BC1 RNA and other ncRNAs in modulating APP in FXS*. Inhibiting the BC1-FMRP association in Tg2576 mice blocks Aβ aggregation and protects against spatial learning and memory deficits whereas overexpression of BC1 induces Aβ peptide accumulation and impairs spatial learning and memory [26]. BC1 RNA has two dendritic targeting codes [126]. It will be of interest to define the potential role of BC1 RNA and other ncRNA in the dendritic localization and translation of *App* mRNA.

4. *Investigate PrP^C in FXS models*. Despite a substantial quantity of work to date regarding PrPC/$mGluR_5$/Aβ signaling, PrP^C has not yet been studied in FXS models. It will be important to examine PrP^C in FXS models and to consider the effects of $mGluR_5$ and APP-related therapeutics on PrP^C, which plays a pivotal role in the signaling pathway.

5. *Quantitate $mGluR_5$ in multiple brain regions and cell types*. Substantial evidence suggests that $mGluR_5$ and downstream signaling molecules are differentially expressed dependent on disease status, brain region and cell type. It would be informative to perform positron emission tomography (PET imaging) in preclinical mouse models and in patients with FXS to determine $mGluR_5$ binding and density in critical brain regions. A $mGluR_5$ tracer has been used to quantitate higher $mGluR_5$ binding potential by PET in postcentral gyrus and cerebellum, but not in other brain regions, of individuals with autism [127]. There was a positive correlation with the lethargy subscale score on the Aberrant Behavior Checklist (ABC) in the precuneus [127]. In the cerebellum, there were

significant negative correlations between binding potential and ABC total score, ABC hyperactivity subscale score, and the ABC inappropriate speech subscale score [127]. PET has also been employed to examine *in vivo* changes in $mGluR_5$ in an Aβ pathology model (Tg-Arc_{SWE} mice) [69]. PET with [^{11}C]ABP688, which is highly specific to $mGluR_5$, resulted in similar levels of tracer in mice regardless of genotype or age and identified receptor-dense brain regions (hippocampus, thalamus, and striatum) [69]. PET with the $mGluR_5$ specific radiotracer ([^{18}F]FPEB) in 5xFAD AD model mice indicated significantly lower radioactivity and binding activity in the hippocampus and striatum of 5xFAD mice (APP transgene with Swedish, Florida and London AD mutations and PS-1 with the M146L and L286V mutations) compared to control animals indicating that $mGluR_5$ is downregulated in the limbic system [73]. It is of interest to quantitate how $mGluR_5$ expression, localization, density and association with PrP^C and Aβ change with development, cell type and disease model

6. *Understand the synergy between FMRP and APP*. For example, J20 AD mice exhibit elevated APP expression but are not very sensitive to AGS [113]. The inclusion of flanking sequences in the human *APP* gene in this model likely alters the temporal and spatial expression of APP and metabolites resulting in a higher seizure threshold. Genetically crossing J20 with $Fmr1^{KO}$ mice produces offspring that overexpress human APP in the context of the $Fmr1^{KO}$ background. Male offspring ($Fmr1^{KO}$/J20) do not exhibit an exacerbated AGS phenotype in comparison to $Fmr1^{KO}$ and unlike FRAXAD mice [111,113]. However, $Fmr1^{Het}$/J20 female mice exhibit 50% wild running and 40% AGS rate in response to audiogenic stimulation, which is significantly higher than WT, $Fmr1^{HET}$ and J20 controls,supporting a dosage effect and the assertion that FMRP works in synergy with APP to regulate hyperexcitability [113].

7. *Study APP synthesis and cleavage in response to environmental stressors*. To date, studies have not been published indicating whether environmental stressors can alter the binding or activities of RBPs or microRNAs that interact with APP mRNA and thus affect protein production. For example, seizures are associated with the consumption of soy-based diets in mouse and human models [112,128]. Gastrointestinal problems are common in FXS and these infants may be more likely to consume alternative diets such as soy-based infant formula. FXS is not part of the newborn screening panel; thus, most children are 2–3 years of age before diagnosis and well past the period of infant diet selection. Thus, it is important to determine if infant diet can affect disease outcomes. The soy phytoestrogen daidzein increases APP expression in primary cultured neurons and increases wild running in response to audiogenic stimulation in WT mice [112].

8. *Investigate $mGluR_5$/FMRP/APP signaling in other FXS models*. Elevated APP causes accelerated generation of neural progenitors and neurons in sh*Fmr1* mESC [129]. It will be of interest to study this signaling pathway in other FXS models such as *Drosophila* and iPSC derived from FXS patients versus controls. Flies that express a 50% reduction in presenilin 1 (PSEN1; a core protein in the

gamma secretase complex involved in APP cleavage) display age related-onset impairment in learning and memory, which can be prevented with MPEP [130].

9. *Employ APP metabolites as exploratory biomarkers in clinical trials*. Drugs under study for FXS such as acamprosate, AFQ056, Donepezil, Ganaxolone, Lithium, Lovastatin, Memantine, Minocycline and Sertraline exhibit on- and/or off-site effects expected to modulate APP, Aβ, BACE1 and/or ADAM10 [116]. In addition, many of the identified receptor and signaling molecules with established roles in FXS are regulated by APP and/or Aβ. A clinical study indicates that acamprosate is associated with a significant reduction in plasma soluble APP and sAPPα in youth with ASD [41]. Youth with FXS-associated ASD exhibited increased sAPPα-processing compared to age-, gender- and IQ-matched youth with idiopathic ASD [41]. Thus, APP metabolites may be promising biomarkers for FXS clinical trials.

10. *Elucidate the connection between FMRP, APP and autism*. There are high levels of APP in children with severely autistic behavior and aggression, and it has been proposed that sAPPα contributes to neuronal overgrowth in autistic brain [131,132]. It will be important to elucidate the connection between FXS, autism and APP.

7. Conclusions

In conclusion, the past decade has produced over 125 papers citing and advancing the 2007 report that $mGluR_5$/FMRP signaling mediates the synthesis of APP. Much remains to be learned in terms of cell, circuit, tissue and disease-specific regulation of APP in FXS models to support development of this potential biomarker and therapeutic target.

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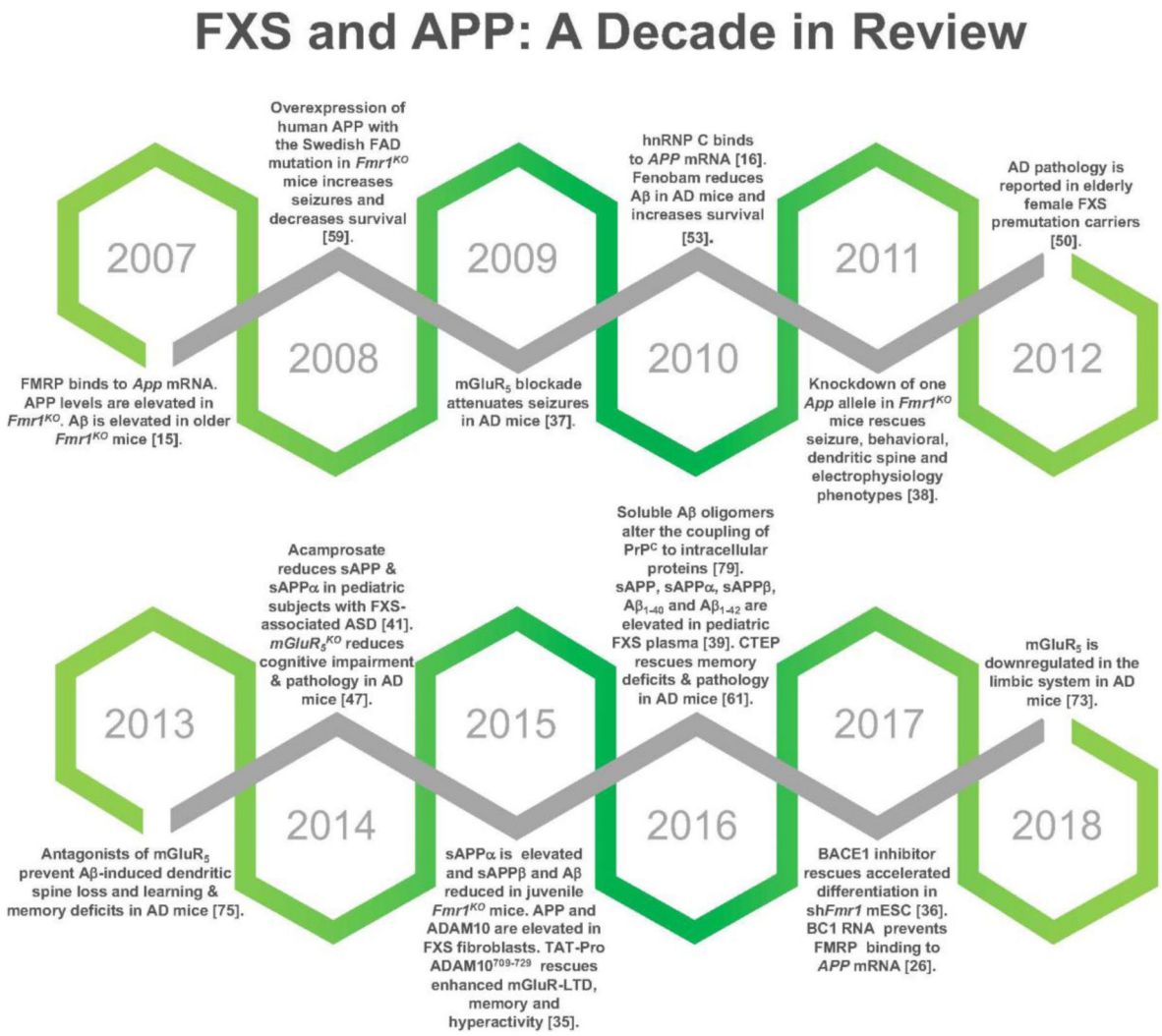

Fig 1.

FXS and APP: A Decade in Review. Major discoveries over the past decade span structural and functional relationships of FMRP and *App* mRNA to the effects of pharmaceutical interventions such as $mGluR_5$ inhibitors on APP metabolite expression and function.

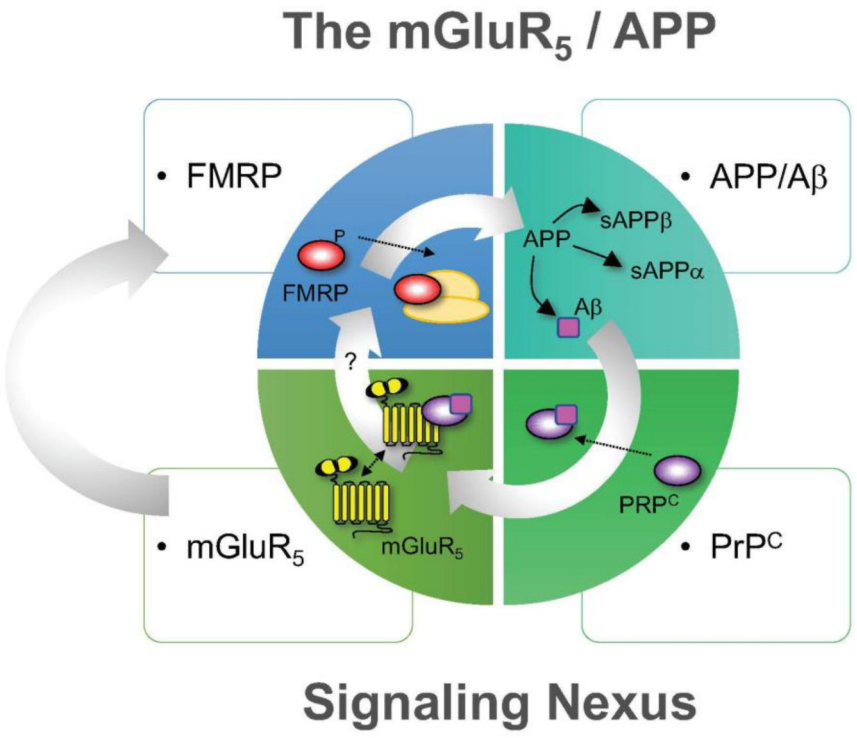

Fig 2.

The $mGluR_5$ / APP Signaling Nexus: The feedback regulation between $mGluR_5$ and APP/Aβ is complex and complicated by the involvement of FMRP and PrP^C. APP synthesis is regulated through a FMRP- and $mGluR_5$-dependent signaling pathway [15]. The phosphorylation status of FMRP regulates its association with actively translating polyribosomes [94]. The generation of APP provides template for cleavage of α-, β- and γ-secretases to produce a multitude of APP metabolites, which have varied functions at the synapse [116]. One of those metabolites, A β, associates with PrP^C, which acts as a bridge between $mGluR_5$ and A β to form a complex that modulates neuronal signaling [75–82]. The potential role of PrP^C in FXS has not been studied. Moving forward, it will be important to explore the temporal and spatial expression and function of APP, APP metabolites, APP secretases, PrP^C, $mGluR_5$ and FMRP in control and FXS models.

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Table 1:

Summary of Cited Alzheimer's Disease Mouse Model Data

Phenotype		3XTg	5XFAD	*APP*^{E693Q}	APP$_{SWE}$/PS1ΔE9	FRAXAD	J20	R1.40	Tg2576	Arc$_{SWE}$
Mutated Genes *		*APP$_{SWE}$ MAPT$_{P301L}$ PSEN1$_{M146V}$*	*APP$_{SWE}$ APP$_{FLOR}$ APP$_{LOND}$ PSEN1$_{M146L}$ PSEN1$_{L286V}$*	*APP$_{DUTCH}$*	*APP PSEN1ΔE9*	*APP$_{SWE}$ Fmr1$_{KO}$*	*APP$_{SWE}$ APP$_{IND}$*	*APP$_{SWE}$*	*APP*	*APP$_{SWE}$ APP$_{Arc}$*
APP Gene Contains UTR Sequences		No [133]	Yes [134]			No [59]	Yes [135]	Yes [136]	No [33]	Yes [137]
Early Mortality					Yes [110]	Yes [59]			Yes [59]	
AGS Susceptibility		Yes [114]				Yes [111]	No [113]	No [113]	Yes [111]	
Anti-Aβ Rescues	**Seizures**	Yes [114]							Yes [111]	
	Epilepti form Discharge Duration	Yes [114]								
mGlu R$_5$ Inhibitors or Genetic Knockout Rescues	**Seizures**	Yes [114]				Yes [37]			Yes [37,111]	
	Aβ Levels or Plaques	Yes [61]			Yes [47,61]			Yes [53]	Yes & No ** [53]	
	Dendritic spine or synapse loss				Yes [75,86]					
	Early Mortality				Yes [110]					
	Epilepti form	Yes [114]								
	Discharge Duration									
	Learning / Memory	Yes [75]			Yes [47,61,75,86]					
Altered Expression of mGluR$_5$			Yes [73]		Yes [47]					
***Fmr1* gene Differentially Spliced**				Yes [103]	Yes [103]					
FMRP Differentially Expressed					No [45] Yes [47]				Yes [46]	

* *APP$_{SWE}$* = APP KM670/671NL (APP with Swedish familial AD mutation); *APP$_{FLOR}$* = *APP* I716V (APP with Florida familial AD mutation); *APP$_{LOND}$* = *APP* V717I (APP with London familial AD mutation); *APP$_{DUTCH}$* = *APP* E693Q (APP with Dutch familial AD mutation); *APP$_{IND}$* = *APP* V717F (APP with Indiana familial AD mutation); *APP$_{ARC}$* = *APP* E693G (APP with Arctic familial AD mutation); *MAPT* P301L (tau familial AD mutation); *PSEN1* (presenilin 1 familial AD mutation).

** Human Aβ produced from the transgene is not reduced. Endogenous mouse Aβ is reduced.