FMRP Mediates mGluR$_5$-Dependent Translation of Amyloid Precursor Protein

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Amyloid precursor protein (APP) facilitates synapse formation in the developing brain, while beta-amyloid (Aβ) accumulation, which is associated with Alzheimer disease, results in synaptic loss and impaired neurotransmission. Fragile X mental retardation protein (FMRP) is a cytoplasmic mRNA binding protein whose expression is lost in fragile X syndrome. Here we show that FMRP binds to the coding region of APP mRNA at a guanine-rich, G-quartet–like sequence. Stimulation of cortical synaptoneurosomes or primary neuronal cells with the metabotropic glutamate receptor agonist DHPG increased APP translation in wild-type but not fmr-1 knockout samples. APP mRNA communoprecipitated with FMRP in resting synaptoneurosomes, but the interaction was lost shortly after DHPG treatment. Soluble Aβ$_{40}$ or Aβ$_{42}$ levels were significantly higher in multiple strains of fmr-1 knockout mice compared to wild-type controls. Our data indicate that postsynaptic FMRP binds to and regulates the translation of APP mRNA through metabotropic glutamate receptor activation and suggests a possible link between Alzheimer disease and fragile X syndrome.

Introduction

Alzheimer disease (AD) is a neurodegenerative disorder characterized by senile plaques and neurofibrillary tangles. The plaques are predominantly composed of beta-amyloid (Aβ), a 39–42 amino acid peptide cleaved from the amyloid precursor protein (APP). APP is likely important for synapse formation in the developing brain [1], while excess Aβ causes impaired synaptic function [2]. Disordered synaptic transmission is also a hallmark of other neuronal disorders, such as epilepsy and fragile X mental retardation syndrome (FXS).

FXS is the most prevalent form of inherited mental retardation, affecting one in 4,000 men and one in 8,000 women. This X chromosome–linked disorder is characterized by moderate to severe mental retardation (overall IQ <70), autistic-like behavior, seizures, facial abnormalities (large, prominent ears and long, narrow face) and macroorchidism [3]. At the neuroanatomic level, FXS is distinguished by an overabundance of long, thin, tortuous dendritic spines with prominent heads and irregular dilations [4,5]. The increased length, density, and immature morphology of dendritic spines in FXS suggest an impairment of synaptic pruning and maturation.

In the majority of cases, FXS results from a trinucleotide (CGG) repeat expansion to >200 copies in the 5′-UTR of the fmr-1 gene (located at Xq27.3) [6]. The CGG expansion is associated with hypermethylation of the surrounding DNA, chromatin condensation, and subsequent transcriptional silencing of the fmr-1 gene, resulting in the loss of expression of fragile X mental retardation protein (FMRP) [7].

FMRP is an mRNA-binding protein that is ubiquitously expressed throughout the body, with significantly higher levels in young animals [8]. The protein has two heterogeneous nuclear ribonucleoprotein (hnRNP) K homology domains and one RGG box as well as nuclear localization and export signals. FMRP interacts with BC1 RNA as well as a number of RNA-binding proteins, including nucleolin and YB1 and the FMRP homologs FXR1 and FXR2 [9]. FMRP has been implicated in translational repression [10–15], and in the brain, cosediments with both translating polyribosomes [16] and with mRNPs [12]. The RGG box of FMRP binds to intramolecular G quartet sequences in target mRNAs [17], while the KH2 domain has been proposed to bind to so-called kissing complex RNAs based on in vitro selection assays [18]. In addition, FMRP binds to uridine-rich mRNAs [19,20]. In aggregate, more than 500 mRNA ligands for FMRP have been identified, many with the potential to influence synaptic formation and plasticity [10,17].

FMRP is required for type 1 metabotropic glutamate receptor (mGluR)-dependent translation of synaptic proteins, including FMRP and postsynaptic density 95 (PSD-95) [21,22]. Both PSD-95 and FMRP mRNAs contain putative G-quartets in their 3′-UTR and coding sequence, respectively [22,23]. Database searches revealed that APP mRNA possesses a G-quartet–like motif in the coding region (position 825–846 of the mouse sequence) embedded within a guanine-rich domain (694–846) containing several DWGG repeats. APP mRNAs (70% of APP695 and 50% of APP751/770) are associated with polyribosomes in rat brain [24], suggesting that translational regulation could play an important role in

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APP production. Indeed, APP contains a 5′-UTR iron response element previously implicated in translation control [25]. Therefore, we hypothesized that APP mRNA translation would be regulated by FMRP.

We now show that after stimulation with the mGluR agonist DHPG, APP levels increased significantly in wild-type (WT) but not synaptoneurosomes (SNs) or cultured neurons from knockout (KO) animals. In KO SNs or neurons, APP was constitutively elevated. APP mRNA coimmunoprecipitated with FMRP in WT, resting SNs, but this interaction was lost with DHPG treatment. FMRP monomer bound to the 5′ end of the G-rich sequence in the coding region of APP mRNA. Our data indicate that FMRP represses the translation of APP through mGluR-dependent interactions with APP mRNA. Consistent with constitutively elevated APP levels, the proteolytic products Aβ40 and Aβ42 are elevated in the brains of fmr-1 KO mice compared to WT.

Results

APP mRNA Coimmunoprecipitates with Anti-FMRP

Our laboratory has shown that FMRP and PSD-95 mRNAs are rapidly translated in mouse primary cortical neurons in response to the type 1 mGluR agonist DHPG [22]. Normal regulation was lost in fmr-1 KO-derived neurons, implicating FMRP in this process. These and other FMRP-regulated mRNAs contain G-quartets, which have been proposed as at least one site of mRNA/FMRP interaction [17]. Database searches of brain mRNAs revealed that the coding region of human, mouse, and rat APP mRNAs contained a G-quartet-like sequence (Figure 1A) within a G-rich domain containing several DWGG repeats (Figure 1B). The putative G-quartet motif in APP mRNA has the potential to form a stable structure containing three guanine planes (Figure S1). FMRP binds to G-rich sequences (so-called G-quartets; consensus site: DWGG-N(0–2)-DWGG-N(0–1)-DWGG-N(0–1)-DWGG, where D is any nucleotide except C and W is A or U) [17] arranged in a planar conformation and stabilized by Hoogsteen-type hydrogen bonds. In the human APP mRNAs, the G-rich region containing the putative G-quartet motif is found in all three splice variants (APP695, APP751, and APP770; 87 nucleotides upstream of the sequence coding for the Kunitz-type protease inhibitor domain, which is missing in APP695). FMRP also binds to kissing complex sites [18], but APP mRNA lacks such a site. Therefore, we prepared cortical lysates as well as SNs from WT mice and immunoprecipitated FMRP. Contrary to a previous report utilizing a different protocol for the preparation of SNs [26], APP mRNA is present in SNs, and reverse transcription (RT)–PCR revealed that the message was brought down with specific, but not control, antisera in cortical lysates as well as SNs (unpublished data). Thus, APP mRNA is a potential target of FMRP, presumably via the coding region putative G-quartet.

FMRP Regulates APP Translation

APP is highly expressed in neurons and dendrites and may promote synaptic maturation [1]. Conversely, overexpression of APP and its proteolytic product, Aβ, have been implicated in the synaptic losses seen early in the development of AD [27]. Therefore, we hypothesized that APP translation was regulated by dendritic FMRP. We utilized fmr-1 KO mice, a rodent model for FXS, that display dendritic spine anomalies similar to that in the human disorder [28–30]. Cortical SNs were prepared from both WT and fmr-1 KO mice, and overall protein synthesis was analyzed in response to DHPG (100 μM)–induced mGluR activation. SNs from either animal were metabolically active with equivalent total 35S-Met incorporation (Figure 2). Therefore, FMRP was not required for basal protein synthesis, which is in agreement with a prior report [31]. However, we did not observe an increase in overall protein synthesis in response to DHPG, whereas Weiler and colleagues [31] observed a 1.3-fold increase in 35S-Met incorporation after 5 min of stimulation.

To assess de novo synthesis, 35S-labeled WT or KO SNs were immunoprecipitated with anti-APP. After 15 min of incubation, untreated WT SNs translated modest amounts of APP, which rapidly increased by 2.7-fold with DHPG treatment. After 1 hr, APP remained elevated in stimulated SNs over the control, but the difference was less (1.6-fold) than at 15 min, suggesting more persistent translation in the stimulated controls, slowing of new synthesis after stimulation, and/or compensatory protein turnover in the DHPG-treated samples (Figure 3A and 3B). In KO SNs, APP synthesis was less than in WT SNs and showed a minimal response to DHPG. The translational inhibitor anisomycin blocked DHPG-mediated synthesis of APP, as did the specific mGluR5 inhibitor MPEP (Figure 3C and 3D).

In order to assess changes in steady-state levels, rather than new protein synthesis, APP was measured in WT and KO SNs in response to DHPG by Western blot analysis (Figure 4). In WT SNs, there was a rapid increase in total APP levels within 5 min of DHPG treatment (1.6-fold, n = 3), which was completely absent in KO SNs. Regardless of treatment, APP levels remained nearly constant over time in KO SNs, as did β-actin. In the absence of DHPG, steady-state levels of APP were substantially higher in KO SNs compared to WT SNs. Within 20 min of DHPG treatment, APP levels in WT SNs approached those seen in unstimulated KO SNs (Figure 4). Protease inhibitors increased steady-state levels of APP in WT SNs to those seen in KO SNs (unpublished data). These data...
suggest that APP mRNA is translationally repressed by FMRP in unstimulated WT SNs. mGluR activation rapidly derepresses APP synthesis as shown for FMRP and PSD-95 [21,22]. APP levels during maximal derepression approach those seen constitutively in \( fmr-1 \) KO cells. After the cessation of mGluR signaling, APP levels presumably drop due to degradation, which appears more robust in WT than KO cells.

FMRP Regulates Dendritic APP Levels in Cultured Neurons

SNs are a relatively crude preparation of pre- and postsynaptic densities that are contaminated with other cell types, such as astrocytes, which form synapses with neurons. Thus, we prepared primary embryonic day–18 cortical neuron cultures from WT and \( fmr-1 \) KO brains and assessed dendritic APP levels by immunofluorescence. APP was found in the cell body as well as dendritic puncta of both WT and \( fmr-1 \) KO neurons (Figure 5A). There was a 21% increase in the basal level of APP in untreated \( fmr-1 \) KO neurons compared to WT (Figure 5B). Neurons stimulated with DHPG for 10 and 20 min prior to cell fixation showed a 18%–25% increase in dendritic APP levels in WT but no increase in \( fmr-1 \) KO cultures (Figure 5B). These data confirm our findings in SNs that (1) \( fmr-1 \) KO mice have higher basal synaptic levels of APP, and (2) DHPG increases APP levels selectively in WT samples. These data also demonstrate that FMRP and mGluR activation regulate APP synthesis in both FVB and C57BL/6 mice, as the SNs were prepared from the former strain, and the primary cortical neurons from the latter strain.

mGluR Activation Does Not Affect APP mRNA Stability

FMRP and homologs have been implicated in the control of mRNA decay. There are increased APP mRNA levels in the cerebral cortex, hippocampus, and cerebellum in a FXS mouse model [32], and FXR1P, an FMRP homolog, is an AU-rich element–binding protein that binds to and regulates TNF\( \alpha \) mRNA stability and translation [33]. APP mRNA contains two 3′-UTR cis-elements within 200 bases of the stop codon that mediate message stability. Hence, we analyzed APP mRNA and 18S rRNA decay in SNs by real-time PCR. APP mRNA did not decay over 120 min regardless of mGluR activation in WT and KO SNs (Figure S2). These data indicate that mGluR-dependent APP translation was independent of mRNA stabilization. APP mRNA has a half-life of approximately 5 h in resting immune cells, which is prolonged in activated cells [34,35] or rat PC12 (Westmark and Malter, unpublished data). Thus, APP mRNA decays with comparable kinetics in SNs and mammalian cells.

mGluR Activation Dislodges APP mRNA from an mRNP Complex Containing FMRP

The mechanism underlying FMRP-mediated translational repression is controversial [36]. Alterations in the association of FMRP with polyribosomes, small nontranslated RNAs, or
other proteins have all been proposed [9,12,37,38]. We asked if the APP mRNA/FMRP interaction changed after DHPG. Thus, FMRP was immunoprecipitated from WT SNs (60 min after DHPG), and the pellet was reverse transcribed and analyzed by real-time quantitative PCR (qPCR). APP mRNA was readily detected in anti-FMRP pellets in untreated WT SNs (Figure 6A). However, APP mRNA associated with FMRP could not be detected in DHPG-stimulated WT SN immunoprecipitates (IPs) or in the KO with or without DHPG within 40 cycles of real-time PCR. The negative controls for this experiment were duplicate IPs in the absence of 7G1–1 FMRP antibody, which also did not produce any real-time PCR Ct values for APP mRNA within 40 cycles (data not shown). The >60-fold difference in FMRP-associated APP mRNA was highly significant. Evaluation at earlier times revealed that the APP mRNA–FMRP complex was lost within 5 min of DHPG treatment (unpublished data).

Immunoprecipitation of FMRP from WT SNs followed by Western blotting (Figure 6B) or 35S-Met incorporation analysis (unpublished data) demonstrated that DHPG treatment does not interfere with the ability of anti–7G1–1 antibody to bind to FMRP. In fact, in both assays there was more FMRP immunoprecipitated from the DHPG-treated WT SNs, which is in agreement with previous reports that DHPG stimulates the dendritic translation of FMRP [22,39]. Our data suggest that physical interactions between FMRP and APP mRNA underlie translational repression, with mGluR activation rapidly moderating these events. Presumably, the loss of FMRP/APP mRNA interaction results in rapid, pulsatile protein expression in dendrites.

**FMRP RNP Binds to G-Rich Sequences**

FMRP is a component of large RNP complexes [38]. The data presented here demonstrate that APP mRNA is also associated with this RNP. To determine the likely interaction site, in vitro RNase protection assays were performed on FMRP IPs from whole-cortex lysates. Residual APP mRNA was mapped by RTqPCR with primers immediately surrounding the predicted G-quartet (Figure 7A). Surprisingly, the G-rich area immediately preceding the G-quartet (nt 699–796) was approximately 4-fold more protected from nuclease digestion than fragments containing the predicted G-quartet (825–846). Although this protected area does not contain a canonical G-
quartet motif, the sequence is very G-rich and contains several closely spaced DWGG repeats. The smallest amplicon (nt 774–871) containing the predicted G-quartet motif amplified a 98-base fragment, of which 46 nucleotides were guanines (47% G-rich; Table S1). Although this is the most G-rich amplicon of those tested, and T1 ribonuclease cuts 3' of single-stranded G-residues, the 98-nt protected fragment (amplicon 699–796) was 40% G-rich, providing nearly equivalent numbers of targets for digestion. Thus, nucleotides 699–796 in the coding region of APP mRNA possess a G-rich sequence that is protected from nuclease digestion by an RNP complex containing FMRP.

The FMRP-containing RNP complex likely protects other cis-elements in APP mRNA as well. Our laboratory has defined a 29-base element located 200 nucleotides downstream of the stop codon in APP mRNA that regulates message decay [35]. We have also identified two proteins, nucleolin and hnRNP C, that bind to this 29-base element [40]. Since nucleolin interacts with FMRP to form multiprotein complexes [38], we would expect the 29-base element to be protected from T1 ribonuclease digestion of anti-FMRP IPs, as shown in Figure 7A (nt 2318–2416). Our data define the G-rich region immediately preceding the predicted G-quartet as the binding site between FMRP and APP mRNA. The loss of FMRP binding at the G-rich region presumably derepresses APP translation, as it was contemporaneous.

Soluble Aβ_{40} and Aβ_{42} Are Increased in fmr-1 KO

Increased translation of APP provides more targets for cleavage by β- and γ-secretases. Therefore, we would expect fmr-1 KO mice to have exacerbated Aβ production with aging. Right-brain hemispheres from middle-aged FVB mice (11–13 mo old) were homogenized in protein extraction buffer containing 1% Triton X-100 and protease inhibitors and the soluble material was analyzed by enzyme-linked immunosorbent assay (ELISA) for Aβ_{40} and Aβ_{42}. The fmr-1 KO mouse brain contained 1.6 times more Aβ_{40} and 2.5 times more Aβ_{42} than WT controls (Figure 8A). We also tested Aβ_{40/42} levels in C57BL/6 mice (12–14 mo old) to ensure that this was not a strain-specific event. We did not observe an increase in soluble Aβ_{40} or Aβ_{42} levels in fmr-1 KO C57BL/6 brain samples (unpublished data), but guanidine-soluble fractions showed a 2.8-fold increase in Aβ_{40} and a 1.2-fold increase in Aβ_{42} (Figure 8B). Therefore, the brains of two distinct murine
strains lacking fmr-1 both showed increased APP and accumulated pathogenic Aβ species over time.

**Discussion**

Synaptic plasticity is required for normal learning and memory and is impaired in FXS. High dendritic spine density is normal for young mice, but synapse pruning during postnatal development is absent in the KO, resulting in increased spine density in adulthood [42]. The molecular basis for defective pruning in fmr-1 KO mice is unknown, but likely reflects the loss of FMRP-regulated translation of synaptic mRNA. FMRP regulates group 1 mGluR-dependent translation of mRNA targets important in diverse neuronal functions [36]. For example, FMRP normally represses the translation of microtubule-associated protein 1B (MAP1B) mRNA during synaptogenesis. In FXS, MAP1B expression is constitutively elevated, leading to abnormally increased microtubule stability [43]. Therefore, it is of great interest to identify FMRP-dependent synaptic mRNAs that contribute to dendritic structure and function.

Herein, we show that APP mRNA is a previously unappreciated target for FMRP-mediated translational repression at the synapse. The normal physiologic role of APP remains ill defined, but increasing evidence suggests an important role in synapse formation [44,45] and maturation [1]. APP localizes to postsynaptic densities, axons, dendrites, and neuromuscular junctions [1,46]. APP/APP-like protein 2 double-KO mice exhibit defective neuromuscular junctions, excessive nerve terminal sprouting, and defective synaptic transmission [47]. APP is developmentally regulated with maximal expression during synaptogenesis and subsequently declines when mature connections are completed [48,49]. Therefore, synaptic overexpression of APP during early development may contribute to the immature dendritic spines and inadequate synaptic pruning characteristic of FXS.

We have identified a G-rich region located within APP mRNA. Figure 6 shows the effect of mGluR activation on FMRP binding to APP mRNA. Figure 7 demonstrates the binding of FMRP to the G-rich region in APP mRNA using immunoprecipitation and RT-qPCR. These experiments support the hypothesis that FMRP represses APP translation, contributing to synaptic pruning defects in FXS.
Figure 8. Increased Aβ40 and Aβ42 Levels in fmr-1 KO Mice

(A) Soluble brain lysates from 1-y-old WT and fmr-1 KO mice (FVB strain) analyzed by ELISA and plotted as a percentage of soluble Aβ compared to WT controls. Student t-tests: p = 0.06 (Aβ40) and p = 0.001 (Aβ42).

(B) GnHCl-soluble brain lysates from 1-y-old WT and fmr-1 KO mice (C57BL/6 strain) analyzed by ELISA and plotted as a percentage of GnHCl-soluble Aβ compared to WT controls. Student t-tests: p < 0.001 (Aβ40) and p = 0.39 (Aβ42).

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We would predict that constitutively upregulated APP would lead to increased processing to Aβ. Indeed, increased Aβ40 and Aβ42 are present in two mouse models for FXS. To date, the only abnormal neuropathologic observations in the human FXS brain have involved impaired synaptic pruning and maturation [51]; however, a very limited number of aged FXS brains have been studied [4,29,52], so other neuropathologies, such as increased amyloid burden and synaptic degeneration normally associated with AD, cannot be excluded. It is difficult to measure cognitive decline in mentally retarded individuals; however, in support of our prediction, fragile X–associated tremor/ataxia syndrome in males is associated with dementia [53].

The normal physiologic function(s) of APP are not well understood, albeit the protein is likely important for synapse formation in the developing brain [1]. A recent report demonstrates that children with severe autism and aggression express 2-fold more secreted βAPP (1,200 pg/ml) than children without autism (500 pg/ml) [54]. Many people with FXS (67% of men and 23% of women) are also autistic [55]. Interestingly, the highest levels of secreted βAPP were found in two children with FXS [54]. Thus, overproduction of secreted βAPP may contribute to the developmental disabilities observed in patients with FXS and autism. In addition, FMRP mRNA and protein expression are downregulated as a function of aging in the mouse brain [56], suggesting that repressed transcripts, such as APP, would be upregulated with aging, a well-known phenomenon in animals and humans.

In conclusion, FMRP represses translation of APP mRNA in dendrites, suggesting a link between two neurodevelopmental disorders, FXS and autism, and a neurodegenerative disease, AD.

Materials and Methods

Materials. The anti-FMRP antibody (mAb7G1–1) [10] was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (http://www.uiowa.edu/~dshbwww). The anti-APP polyclonal antibody (catalog number 51–2700) was purchased from Zymed Laboratories (http://www.invitrogen.com), and the anti-mouse β-actin antibody (catalog number A5441), protease inhibitor cocktail (catalog number P2714), ribonuclease T1 (catalog number R1003), and poly(D)-lysine

nucleotides 699–796 in the coding region of APP mRNA as an FMRP-binding site. The G-quartet–like sequence immediately downstream of this G-rich region was not protected from nuclease digestions of FMRP IPs. This result was surprising because the intramolecular G-quartet motif has been identified by in vitro RNA selection assays as the site of interaction with FMRP [17]. As expected from the FMRP interaction site mapping results, alignment of the G-rich region (nt 699–796) and DWGG repeats of mouse APP mRNA is highly conserved with both the human (86%) and rat (93%) sequences. Our data suggest that there may be flexibility in the spacing of the DWGG repeats for G-quartet formation and agrees with previous findings that the presence of a G-quartet does not ensure binding by FMRP [17].

We have determined that FMRP associates with APP mRNA in SN preparations, and that translation of APP mRNA increases in response to DHPG. DHPG-upregulated translation of APP can be blocked by the translational inhibitor anisomycin or the mGluR5-specific inhibitor MPEP. mGluR-mediated translation is concurrent with FMRP dissociation from APP mRNA and is independent of mRNA decay. The rapid dissociation of FMRP from APP mRNA, in response to mGluR activation, suggests that post-translational modifications, such as phosphorylation, may regulate FMRP binding activity. Ceman and colleagues have shown that FMRP is phosphorylated N-terminal to the RGG box and that phosphorylation/dephosphorylation status of the protein is correlated with binding to stalled versus active polyribosomes [50]. Our data support a model developing in the literature whereby FMRP acts as an immediate early-response protein that regulates translation at the synapse. When FMRP is bound to APP or other synaptic mRNAs, translation is repressed. Upon mGluR activation, FMRP is released from the nontranslating RNP, resulting in prompt protein synthesis. In FXS, high levels of protein are constitutively produced that are normally translationally repressed by FMRP.

We would predict that constitutively upregulated APP would lead to increased processing to Aβ. Indeed, increased...
A monoclonal antibody (mAB348) was acquired from Chemicon (http://www5.amershambiosciences.com). Anti-22C11 APP antibody (mAb51) was acquired from Zymed (catalog number 51–2700). Fresh protein A magnetic beads were added and mixed overnight at 4°C. The beads were washed three times with IP buffer, and the final, washed pellets were suspended in 40 μl 2X SDS sample buffer and boiled for 5 min; the proteins were then separated on 12% SDS gels. The gels were transferred to nitrocellulose membrane, dried, exposed to a phosphorimager screen, and scanned on a STORM 860 phosphorimager (Molecular Dynamics, http://www6.amershambiosciences.com). The 120-kDa APP bands were quantitated with ImageQuant software (GE Healthcare Life Sciences, http://www4.amershambiosciences.com).

For the FRET experiments, SNs were incubated with 25 μl anisomycin (40 μM final concentration) or MPEP (10 μM final concentration) for 10 min prior to the addition of 25 μl 35S-Met for 5 min and stimulation with DHPP (100 μM final concentration) for 15 min. Samples were processed as described in the preceding paragraph.

Western blot analysis. Aliquots of SNs were collected at 5, 10, and 20 min after DHPP treatment, quenched with an equal volume of 2X sample buffer (8% SDS, 24% glycerol, 100 mM Tris [pH 6.8], 4% Me2SO), and 0.05% bromophenol blue in an ice-cold buffer containing 20% MeOH with a Criterion Blotter (Bio-Rad, http://www.bio-rad.com; 100 V at 4°C for 75 min). The membranes were stained with rabbit anti-mouse FMRP antibody (dilution, 1:1000), goat anti-rabbit IgG antibody (dilution, 1:2000) followed by hybridization with anti-rabbit or antimouse HRP-conjugated secondary antibodies (dilution, 1:2000). Proteins were visualized by enhanced chemiluminescence on a STORM 860 phosphorimager.

Neuronal cell culture, confocal microscopy, and image analysis. Pregnant females (embryonic day 18) were anesthetized with halothane prior to decapitation and transfer of the uterine sac to ice-cold HBSS. Cortices were removed, washed with ice-cold HBSS, lysed with 0.5 mg/ml trypsin for 25 min at 37°C, washed with HBSS, suspended in NeuroBasal medium (supplemented with 2% B27 supplement, penicillin/streptomycin, and 0.5 mM glutamine), triturated 70X with a 10-ml pipet, and passed through a 70-μm cell strainer. Cells were counted by trypan blue dye exclusion and plated at 1.25 × 105 cells/ml on poly(D)-lysine–coated glass coverslips in 12-well tissue-culture dishes and cultured for 11 d at 37°C/5% CO2. Half of the culture medium was removed and replaced with fresh, warm medium on day 4.

Neuronal cells were treated with 100 μM DHPP, washed with PBS containing 2% FBS, fixed in 4% PFA for 10 min at room temperature, and permeabilized with MeOH (~20°C) for 15 min. Fixed, permeabilized cells were stained with anti-22C11 against the amino-terminus of APP (Chemicon number mA3848; 1:2000 for 1 h) and visualized with goat anti-mouse rhodamine-conjugated secondary antibody (Invitrogen; 1:500 for 30 min in the dark). All washes and antibody dilutions were in PBS containing 2% FBS. Coverslips were fixed to slides with 12 μl ProLong Gold Antifade with DAPI (Invitrogen) and dried overnight.

Images were acquired with a Nikon C1 laser-scanning confocal microscope with ECLIPSE EZ-C1 v2.20 software (Nikon, http://www.nikon.com) at 60X magnification. APP levels in the puncta of four to seven dendrites per sample were quantitated with IMAGE J software using the Analyze Particles function (minimum of 205 puncta analyzed per treatment) (Rasband, W.S., Image J, U.S. National Institutes of Health, http://rsb.info.nih.gov/ij; 1997–2006). Figures were assembled with Adobe Photoshop 8.0 (Adobe Systems, http://www.adobe.com). All DHPP-treated and fmr-1 KO samples were highly statistically different from untreated WT samples by t-test analyses (p < 0.001) and expressed as SEM.

APP mRNA measurements. Aliquots of SNs were collected at the indicated timepoints and flash frozen at ~80°C. The samples were thawed and vortexed to prepare SN lysates. To directly reverse-transcribe RNA from SN lysates without an RNA purification step, a modified method for the detection of mRNA in single neurons was utilized (Brown et al., 2007). Briefly, SN lysates were added per standard RT reaction containing RNase-free DNase I and random nonamer primer. The reactions were incubated at 37°C for 15 min to destroy any contaminating genomic DNA, 65°C for 5 min to inactivate the
DNase I, and 20 °C for 10 min to anneal the random primer. Omniscript RT was added and reverse transcription proceeded at 37 °C for 60 min before inactivation at 93 °C for 5 min. The RT reactions were diluted 5-fold with DEPC water prior to real-time PCR analysis. For the statistical analysis, APP mRNA levels from triplicate experiments were determined, normalized to 18S rRNA, and plotted as a percentage of total APP mRNA. Real-time PCR reactions were optimized for primer and template concentrations and contained 500 nM APP primers (forward: 1701-cctgggacccttttg-1717; and reverse: 1774-gggcggggtcaca-1760) or 300 nM 18S primers (forward: 98-cattaaatcaggtaggedtctttgg-123; and reverse: 181-tgcttaggtgctttgc-155), 0.25 μl SYBR green at a concentration of 10 μM in a 25 μl reaction volume. The cycle conditions were as follows: 2 min at 50 °C and 10 min at 95 °C (40 cycles: 15 s at 95 °C, 1 min at 60 °C), followed by a dissociation stage for 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. The average PCR efficiencies for the APP and 18S primers over a 200-fold concentration range were 100% (APP) and 101% (18S) (n = 9 experiments each), with a delta slope of 0.079. As the difference in slopes between the sample PCR (APP) and the normalization control (18S) was less than 0.1, the comparative C_{T} method was utilized to calculate the relative concentration of APP mRNA levels normalized to 18S rRNA. SN templates were analyzed for genomic DNA contamination. Control RT reactions on SN templates in the absence of reverse transcriptase were analyzed by real-time PCR and found void of APP PCR product. The final APP and 18S PCR products were analyzed by agarose gel electrophoresis and were single bands of the correct molecular weight (74 bp for APP; 84 bp for 18S).

**FMRF IPs and real-time PCR analysis**

SN lysates were preclarified with protein A magnetic beads and immunoprecipitated with 10 μl RNasin, 10 μg 7G1–1 FMRF antibody (or no antibody controls), and 100 μl packed fresh Protein A magnetic beads for 3 h at 4 °C. The IPs were washed with IP buffer (10 mM HEPES [pH 7.4], 200 mM NaCl, 30 mM EDTA [pH 8], and 0.5% Triton X-100) and suspended in 1 ml TRI-Reagent. Total RNA was isolated and precipitated in the presence of 2 μg tRNA. The final pellet was washed in DEPC water, heated for 10 min at 60 °C, and reverse transcribed with Qiagen Omniscript and random nonamer primer (60 min at 37 °C, 5 min at 93 °C). The cDNA was diluted 5-fold and analyzed for APP by qPCR as described immediately above.

**Preparation of whole-cortex lysate**

The cortices from six WT mice (13 d old) were torn into pieces and homogenized in cold immunoprecipitation buffer (10 mM HEPES [pH 7.4], 200 mM NaCl, 30 mM EDTA [pH 8], and 0.5% Triton X-100) containing 2% protease inhibitor cocktail and 0.4 U/ml RNAsin. The homogenate was spun at 1,000 g for 10 min at 4 °C to remove nuclei and unlysed cells, and the pellet was discarded. The cleared lysate was flash frozen in aliquots at −80 °C.

**Ribonuclease T1 digestions and modified CLIP assay**

Pellets from anti-FMRP immunoprecipitations of whole-cortex lysate were washed once with immunoprecipitation buffer and once with DPBS before digestion with ribonuclease T1 (0.8–1.0 U) in a 100-μl reaction volume for 30 min at 37 °C with occasional mixing to disperse the magnetic protein A beads. The digested samples were washed twice with DPBS to remove RNA fragments. Protected RNA was isolated with TRI-Reagent and analyzed by RTqPCR. The primer sequences for the real-time PCR are listed in Table S2. The delta C_{T} between undigested and T1-digested samples was calculated and plotted as a percentage of APP_{mRNA} mRNA.

For the modified CLIP assay [41], cleared cortical lysate was cross-linked with 400 μm 1,10-phenanthroline in an UV Stratamax 2400 (Stratagene, www.stratagene.com) and reverse transcribed with anti-FMRP, and digested with ribonuclease T1. The washed pellets were suspended in 40 μl SDS loading buffer containing no reducing agents, heated for 10 min at 70 °C, applied to a 12% SDS/PA gel, and transferred to 0.45 μm nitrocellulose membrane in Towbin buffer. Western blotting of a duplicate membrane indicated that FMRP migrated as a 80 kDa band encompassing approximately 75–85 kDa molecular weight range was excised, transferred to TRI-Recagent, and vortexed vigorously for 15 min at 37 °C. RNA was isolated and analyzed by RTqPCR.

**Figure S1. G-Quartet Model**

A model for the putative G-quartet sequence located in the coding region of APP mRNA. Canonical G-quartets form two guanine planes, while the putative APP G-quartet has the potential to form three guanine planes.

**Supporting Information**

**Table S1. G-Richness of APP Amplicons**

For the gene products mentioned in this paper are human APP mRNA (NM_000484), mouse APP mRNA (X59379), rat APP mRNA (X07648), and 18S mRNA (M27358).

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References

1. Akaaboun M, Allinquant B, Farza H, Roy K, Magoul R, et al. (2000) Abnormal dendritic spine characteristics in patients with fragile-X syndrome. J Neurosci Res 37: 925–937.

2. Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, et al. (2003) APP processing and synaptic function. Neuron 37: 925–937.

3. Hagerman RJ, Hagerman PJ (2002) Physical and behavioral phenotype of fragile X syndrome: The roots of fragile X syndrome. Nat Rev Neurosci 3: 107–117.

4. Tacchetti C, Serrano-Padron E, Makino S, Leng L, Greenough WT, et al. (2004) Alternate translation of APP reveals deficits in synaptic regulation and inhibitory neurotransmission in fragile X mice. J Neurosci 24: 9114–9125.

5. Wang P, Yang G, Mosier DR, Chang P, Zaidi T, et al. (2005) Defective amyloid precursor protein mRNA decay. Brain Res Mol Brain Res 90: 193–205.

6. Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA, et al. (2001) The fragile-X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. Cell 107: 477–487.

7. Wang P, Yang G, Mosier DR, Chang P, Zaidi T, et al. (2005) Defective amyloid precursor protein mRNA decay. Brain Res Mol Brain Res 90: 193–205.

8. Darnell JC, Fraser CE, Mostovetsky O, Stefani G, Jones TA, et al. (2005) Evidence that fragile X mental retardation protein is a negative regulator of translation. Hum Mol Genet 14: 329–338.

9. Li Z, Zhang Y, Ku L, Wilkinson KD, Warren ST, et al. (2001) The fragile-X syndrome protein FMRP associates with polyribosomes as an mRNP, and the I304N mutation of fragile X mental retardation protein affects recognition and binding to polyribosomes. J Biol Chem 276: 5750–5756.

10. Li Z, Zhang Y, Ku L, Wilkinson KD, Warren ST, et al. (2001) The fragile-X syndrome protein FMRP associates with polyribosomes as an mRNP, and the I304N mutation of fragile X mental retardation protein affects recognition and binding to polyribosomes. J Biol Chem 276: 5750–5756.

11. Wang P, Yang G, Mosier DR, Chang P, Zaidi T, et al. (2005) Defective amyloid precursor protein mRNA decay. Brain Res Mol Brain Res 90: 193–205.

12. Darnell JC, Fraser CE, Mostovetsky O, Stefani G, Jones TA, et al. (2005) Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein FMRP and brain polyribosomes. Genes Dev 19: 903–918.

13. Chen L, Yuen SW, Seto J, Liu W, Toth M (2003) The fragile X mental retardation protein binds and regulates a novel class of mRNAs containing U-rich target sequences. Neuroscience 126: 1005–1017.

14. Doldhanskaay N, Sung YJ, Conti J, Currie JR, Denman RB (2003) The fragile X mental retardation protein interacts with U-rich RNAs in a yeast three-hybrid system. Biochem Biophys Res Commun 305: 434–441.

15. Greenough WT, Klintsova AY, Irwin SA, Galvez B, Bates KE, et al. (2001) Synaptic regulation of protein synthesis and the fragile X protein. Proc Natl Acad Sci U S A 98: 7101–7106.

16. Todd PK, Mack JK, Maler JS (2005) The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent synaptogenesis and synaptic function. EMBO J 24: 4803–4813.

17. Schaeffer C, Bordoni B, Mandel JL, Ehresmann B, Ehresmann C, et al. (2001) The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. EMBO J 20: 4803–4813.

18. Deacon M, Potempska A, Wolfe G, Ramakrishna N, Miller DL (1991) Distribution and activity of alternatively spliced Alzheimer amyloid peptide precursor and scrapie PrP mRNAs on rat brain polymers. Arch Biochem Biophys 288: 29–38.

19. Reuters JT, Randic M, Cahill CM, Eder PS, Huang X, et al. (2002) An iron-responsive element type II in the 5′-untranslated region of the Alzheimers amyloid precursor protein transcript. J Biol Chem 277: 45518–45528.

20. Sung YJ, Weiher II, Greenough WT, Denman RB (2004) Selectively enriched mRNAs in rat synaptosomes. Brain Res Mol Brain Res 127: 81–88.

21. Lacor PN, Buniel MC, Zhang Y, Ku L, Wilkinson KD, et al. (2002) Fragile X mental retardation protein is necessary for neurotransmitter-mediated protein translation at synapses. Proc Natl Acad Sci U S A 101: 17304–17309.

22. D’Agostino V, Warren ST, Zhao W, Torre ER, Alkon DL, et al. (2002) Gene expression profiles in a transgenic animal model of fragile X syndrome. Neurobiol Dis 10: 211–218.

23. Garzon J, Lachance C, Di Marco S, Hel Z, Marzo D, et al. (2005) Fragile X-related protein FXR1 regulates the expression of messenger RNA at the post-transcriptional level. J Biol Chem 280: 5750–5763.

24. Westmark CJ, Maler JS (2001) Extracellular-regulated kinase controls beta-amyloid precursor protein mRNA decay. Brain Res Mol Brain Res 90: 195–201.

25. Zaidi SH, Maler JS (1994) Amyloid precursor protein mRNA stability is controlled by a 29-bp element in the 3′-untranslated region. J Biol Chem 269: 24007–24015.

26. Bear MF, Huber KM, Warren ST (2004) The mGluR theory of fragile X mental retardation. Trends Neurosci 27: 370–377.

27. Feng Y, Abscher D, Eberhart DF, Brown V, Maler HE, et al. (1997) FMRP associates with polyribosomes as an mRNP, and the 150N mutation of fragile X mental retardation protein affects its binding to polyribosomes. J Biol Chem 272: 17292–17298.

28. Ceman S, Brown V, Warren ST (1999) Isolation of an FMRP-associated messenger ribonucleoprotein particle and identification of nucleolin and the fragile X-related proteins as components of the complex. Mol Cell Biol 19: 2039–2052.

29. Weiher II, Irwin SA, Klintsova AY, Spencer CM, Brazelton AD, et al. (1997) Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proc Natl Acad Sci U S A 94: 5394–5399.

30. Galvez R, Greenough WT (2005) Sequences of abnormal dendritic spine development in a fragment mouse cortex reveal the cortex as a model for impaired translation of FMRP-dependent mRNAs. Proc Natl Acad Sci U S A 102: 15201–15206.

31. Li Z, Zhang Y, Ku L, Wilkinson KD, Warren ST, et al. (2001) The fragile X mental retardation protein inhibits translation via interacting with mRNA. Nucleic Acids Res 29: 22762–22833.

32. Mason R, Hoef MG, Trebily S, Filion C, Labelle Y, et al. (2002) Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression. Hum Mol Genet 11: 3007–3017.

33. Rutter J, Perrett RD, Frackowiak RS, Thompson PK, Cunningham VJ, et al. (1995) Dendritic spine abnormalities in the occipital cortex of C57BL/6 Fmr1 knock out mice. Hum Brain Mapp 1: 140–153.

34. Tanaka Y, Greenough WT (2005) Abnormal dendritic spine morphology in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination. Am J Med Genet 98: 161–167.

35. McKinney BC, Grossman AW, Elieoumsa NM, Greenough WT (2005) Dendritic spine abnormalities in the occipital cortex of 5C7BL/6 Fmr1 knockout mice. Am J Med Genet B Neuropsychiatr Genet 136: 98–102.

36. Deane JF, Spanger CC, Klimova AY, Grossman AW, Kim SH, et al. (2004) Fragile X mental retardation protein is necessary for neurotransmitter-activated protein translation at synapses. Proc Natl Acad Sci U S A 101: 17304–17309.

37. Hagerman RJ, Hagerman PJ (2004) Fragile X-associated tremor/ataxia syndrome (FXTAS). Ment Retard Dev Disabil Res Rev 10: 25–30.

38. Slobodnik Jr, Chen D, Farb DA, Munz DW, Maloney B, et al. (2006) High levels of Alzheimer beta-amyloid precursor protein (APP) in children with severe autistic behavior and aggression. J Child Neurol 21: 444–449.

39. Clifford S, Dissanayake C, Bui QM, Huggins R, Taylor AK, et al. (2006) FMRP Regulates APP Translation
Autism spectrum phenotype in males and females with fragile X full mutation and premutation. J Autism Dev Disord. In press.

56. Singh K, Gaur P, Prasad S (2006) Fragile X mental retardation (Fmr-1) gene expression is down regulated in brain of mice during aging. Mol Biol Rep, Epub ahead of print.

57. Dunkley PR, Heath JW, Harrison SM, Jarvie PE, Glenfield PJ, et al. (1988) A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: Homogeneity and morphology of subcellular fractions. Brain Res 441: 59–71.

58. Bagni C, Mannucci L, Dotti CG, Amaldi F (2000) Chemical stimulation of synaptosomes modulates alpha-Ca2+/calmodulin-dependent protein kinase II mRNA association to polysomes. J Neurosci 20: RC76.

59. Comer AM, Gibbons HM, Qi J, Kawai Y, Win J, et al. (1999) Detection of mRNA species in bulbospinal neurons isolated from the rostral ventrolateral medulla using single-cell RT-PCR. Brain Res Brain Res Protoc 4: 367–377.

60. Costantini C, Weindruch R, Della Valle G, Puglielli L (2005) A TrkA-to-p75NTR molecular switch activates amyloid beta-peptide generation during aging. Biochem J 391: 59–67.