Cerebral Protein Synthesis in a Knockin Mouse Model of the Fragile X Premutation

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Abstract

The (CGG)n-repeat in the 5′-untranslated region of the fragile X mental retardation gene (FMR1) gene is polymorphic and may become unstable on transmission to the next generation. In fragile X syndrome, CGG repeat lengths exceed 200, resulting in silencing of FMR1 and absence of its protein product, fragile X mental retardation protein (FMRP). CGG repeat lengths between 55 and 200 occur in fragile X premutation (FXPM) carriers and have a high risk of expansion to a full mutation on maternal transmission. FXPM carriers have an increased risk for developing progressive neurodegenerative syndromes and neuropsychological symptoms. FMR1 mRNA levels are elevated in FXPM, and it is thought that clinical symptoms might be caused by a toxic gain of function due to elevated FMR1 mRNA. Paradoxically, FMRP levels decrease moderately with increasing CGG repeat length in FXPM. Lowered FMRP levels may also contribute to the appearance of clinical problems. We previously reported increases in regional rates of cerebral protein synthesis (rCPS) in the absence of FMRP in an Fmr1 knockout mouse model and in a FXPM knockin (KI) mouse model with 120 to 140 CGG repeats in which FMRP levels are profoundly reduced (80%–90%). To explore whether the concentration of FMRP contributes to the rCPS changes, we measured rCPS in another FXPM KI model with a similar CGG repeat length and a 50% reduction in FMRP. In all 24 brain regions examined, rCPS were unaffected. These results suggest that even with 50% reductions in FMRP, normal protein synthesis rates are maintained.

Keywords
Fmr1, FMRP, fragile X premutation, fragile X syndrome, FXTAS, protein synthesis

Introduction

The (CGG)n-repeat sequence in the 5′-untranslated region of the fragile X mental retardation gene (FMR1) on Xq27.3 is polymorphic. In the normal population, the repeat sequence length is 5 to 54 and the gene product, fragile X mental retardation protein (FMRP) has normal expression (Fu et al., 1991). Individuals with fragile X syndrome (FXS), the most common form of hereditary intellectual disability and monogenic cause of autism, have more than 200 CGG repeats resulting in gene methylation, transcriptional silencing, and absence of FMRP (Verkerk et al., 1991). This is known as the full mutation. Individuals with (CGG)n between 55 and 200 are known as fragile X premutation (FXPM) carriers. The gene remains unmethylated in these individuals and transcription is not silenced, but there is a high risk of expansion to a full mutation on maternal transmission. Because the prevalence of FXPM in the general population, approximately 1/290 males and 1/148 females (Maenner et al., 2013), is much higher than that of FXS, FXPM may have a greater impact on the general population than FXS.

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FXPM carriers are at increased risk for developing progressive neurodegenerative syndromes, including fragile X-associated tremor/ataxia syndrome (FXTAS; Hagerman et al., 2001) and fragile 
X-associated primary ovarian insufficiency (FXPOI) in females (Allingham-Hawkins et al., 1999). Further evidence suggests that FXPM carriers have a higher incidence of neuropsychological symptoms (Cornish et al., 2005, 2009; Hunter et al., 2008; Kogan et al., 2008). These features are thought to be caused by a toxic gain of function of elevated levels of CGG repeat-containing FMR1 mRNA (Jin et al., 2003; Hessl et al., 2005; Brouwer et al., 2008a; Hagerman, 2013). As FMRP levels moderately decrease with increasing CGG repeat length, CGG repeat knockin (KI) models have been generated in mice (Bontekoe et al., 2001; Entezam et al., 2007). In one (KI_Dutch), a region of the Fmr1 gene including the endogenous repeat tract (CGG)_8 was replaced with a cloned human premutation allele (CGG)_{98} (Bontekoe et al., 2001). The repeat tract in the other model (KI_NIH) was generated by serial ligation of short, stable CGG-CCG repeats (Grabczyk and Usdin, 1999; Entezam et al., 2007). As in the human disease, the KI mice in both models exhibit repeat-length instabilities on transmission to succeeding generations and a direct relationship between repeat number and Fmr1 mRNA levels in brain (Bontekoe et al., 2001; Entezam et al., 2007). KI mice also display behavioral phenotypes similar to those seen in human FXPM individuals, such as motor deficits, anxiety, and impairments of learning and memory (Van Dam et al., 2005; Hunsaker et al., 2009, 2011, 2012; Qin et al., 2011; Diep et al., 2012). FMRP levels tend to decrease with increasing CGG repeat length, and in both KI models, brain FMRP levels are decreased; the effect size varies across studies (Willemsen et al., 2003; Brouwer et al., 2007, 2008a, 2008b; Entezam et al., 2007; Qin et al., 2011; Berman et al., 2012; Ludwig et al., 2014). Previously, we studied young adult, male KI_NIH PM mice with CGG_\text{120-140} and found that they had a very similar behavioral phenotype to Fmr1 knockout (KO) mice (Qin et al., 2011). We found that young adult KI_NIH mice exhibited hyperactivity in the open field, less general anxiety in both the open field and elevated zero maze, impaired learning and memory on a passive avoidance test, and subtle deficits on a test of social interaction. Motor learning was normal as assessed by the rotarod test. In our study, KI_NIH mice had twofold and sixfold increases in Fmr1 mRNA and drastic decreases in levels of FMRP; FMRP levels in whole brain were reduced to 15% of wild type (WT). Additionally, regional rates of cerebral protein synthesis (rCPS) measured in vivo were statistically significantly increased similar to our finding in Fmr1 KO mice (Qin et al., 2005), suggesting that the decreased concentration of FMRP may contribute to the KI_NIH phenotype. Our goal in the present study was to test how the expanded CGG repeat sequence in Fmr1 affects rCPS in a model (KIDutch) in which FMRP levels are reported to be either not affected (Willemsen et al., 2003) or moderately reduced (Brouwer et al., 2008a; Berman et al., 2012).

Materials and Methods

Animals
The KI_Dutch mice generated by exchanging the murine endogenous (CGG)_8 with a human (CGG)_{98} (Bontekoe et al., 2001) were obtained from Erasmus Medical College. The genetic background was C57BL/6. Animals for these experiments were bred in-house. Briefly, WT and KI_Dutch mice were produced by pairing female mice heterozygous for the KI_Dutch allele with WT males. This strategy yielded WT and KI_Dutch male mice in the same litters. Mice were group housed in a central facility and maintained under controlled conditions of normal humidity and temperature with standard alternating 12-hr periods of light and darkness. All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee.

Determination of CGG Repeat Size

DNA was extracted from mouse tail with Gentra Puregene Mouse Tail Kit (Puregene, Gentra Systems, Inc., Minneapolis, MN, USA) and kept in Tris-EDTA (TE) buffer. The size of the CGG repeat tract was monitored by polymerase chain reaction (PCR) with the primers \text{CGG-F (5'-CGGAGGCGCCGCTGCCAGG-3') and CGG-R (5'-TGCGGCGCGCGCTGAGGGCCAG-3')} as described previously (Hukema and Oostra, 2013).

Western Blotting

WT and KI_Dutch mice were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and decapitated. The cerebellum and cerebrum were separated and frozen in dry ice. Frozen sections, 100 \mu m in thickness, were prepared at −22°C (Leica 1850 cryostat; Leica Microsystems, Deerfield, IL), and 11 regions were punched by means of Harris Uni-Core (1.25 mm; Electron Microscopy Sciences, Hatfield, PA). Regions were weighed and homogenized in 1% (w/v) ice-cold tissue protein
extraction reagent (T-PER) (Thermo Scientific, Rockford, IL) with 1% Halt protease inhibitor cocktail (Thermo Scientific) and 1% phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged (12,000 × g, 4°C, 15 min). The supernatant fractions were collected as protein samples, which were separated by NuPAGE on Bis–Tris gels and subjected to Western blotting with a WesternBreeze Chemiluminescent kit (Invitrogen, Carlsbad, CA). FMRP was detected with a rabbit polyclonal antibody to FMRP, ab17722 (Abcam Inc, Cambridge, MA). Protein extracts from the brains of Fmr1 KO animals were used as negative controls. Blots were quantified by densitometry with MCID Analysis system (Interfocus Imaging Ltd, Linton, Cambridge, UK).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH rabbit monoclonal antibody; Cell Signaling Technology, Danvers, MA) was used as a loading control.

### Regional Rates of Cerebral Protein Synthesis (rCPS)

We used the autoradiographic L-[1-14C] leucine method to determine rCPS in young adult WT (n = 7) and KI_Dutch (n = 7) mice as described previously (Qin et al., 2005). Briefly, mice under light isoflurane anesthesia were prepared for studies by insertion of polyethylene catheters into a femoral artery and vein. Mice recovered from the surgery overnight and were permitted to move freely. The experimental period was initiated by an intravenous pulse injection of 100 μCi/kg of L-[1-14C]leucine (specific activity, 60 mCi/mmol; Moravek Biochemicals, Brea, CA). Timed arterial samples were collected during the following 60 min for determination of the time courses of plasma concentrations of leucine and [14C]leucine. At the end of the experimental interval, brains were removed, frozen, and serial sections, 20 μm in thickness, were prepared for quantitative autoradiography. Autoradiograms were digitized (MCID Analysis, Interfocus Imaging Ltd, Linton, Cambridge, UK), the concentration of 14C in each region of interest was determined, and rCPS was calculated by means of the operational equation of the method (Smith et al., 1988). The value of lambda in the equation was 0.603 (Qin et al., 2005). Brain regions were identified by reference to a mouse brain atlas (Paxinos and Franklin, 2001).

### Statistical Analysis

Data are expressed as mean ± SEM. Statistically significant interactions were analyzed by repeated measures (RM) analysis of variance (ANOVA). Data from Western blots were analyzed for differences between WT and KI_Dutch by means of one-tailed Students’ t-tests. The criterion for statistical significance was p ≤ .05. We used SPSS (IBM, Armonk, NY) for statistical analyses.

### Results

**FMRP Levels in Brain are Negatively Correlated With Number of CGG Repeats**

We determined the number of CGG repeats and whole brain levels of FMRP in 10 KI_Dutch mice. The number of CGG repeats ranged from 114 to 279, and FMRP levels decreased from 59% to 14% of the mean of three WT values (Figure 1(a) and (b)). We found a statistically

![Figure 1](image-url)

**Figure 1.** Effects of CGG repeat insertion on FMRP in brain regions. (a) Change in FMRP/GAPDH in whole brain as % of WT controls. KI_Dutch mice had CGG repeat lengths ranging from 119 to 279. The negative correlation (Pearson correlation coefficient, r = -0.6571) is statistically significant (p < .002). The WT control FMRP/GAPDH is the mean of two determinations (0.464, 0.481). (b) Western blot of whole brain extracts from WT and KI_Dutch mice with CGG repeats range 119 to 279. (c) Change in FMRP/GAPDH ratios in whole brain and 12 subregions as % of WT controls. KI_Dutch mice had CGG repeats range 119 to 151. Bars are the mean ± SEM determined in three mice of each genotype. We used a rabbit polyclonal antibody to FMRP (ab17722). FMRP = fragile X mental retardation protein; WT = wild type; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; CX = cortex.
Unaltered rCPS in KI\textsubscript{Dutch} Mice

At the time of rCPS determination, physiological variables in WT and KI\textsubscript{Dutch} mice were well matched with respect to age, body weight, and other physiological variables measured (Table 1).

To compare the effects of the expanded CGG repeat in KI\textsubscript{Dutch} mice to its effects in KI\textsubscript{NHL} mice, we analyzed rCPS in the same 19 regions studied in our previous study with the addition of motor cortex and stratum radiatum of the dorsal and ventral hippocampus. In the present study, we determined rCPS in 21 brain regions including the dorsal hippocampus as a whole, the ventral hippocampus as a whole, four subregions of the dorsal and ventral hippocampus, six cortical areas, and five other regions (Figures 2 and 3). Results of rCPS determinations were analyzed by means of RM ANOVA with brain region as the within-subjects factor and genotype as the between-subjects factor. The interaction between region and genotype was not statistically significant, $F(2.7, 18.9) = 1.154$, $p = .35$, and there was no main effect of genotype, $F(1, 7) = 0.676$, $p = .44$. As expected from previous studies in which we measured rCPS \textit{in vivo}, we did find a statistically significant effect of region, $F(2.7, 18.9) = 204.86$, $p < .001$, indicating that rCPS varies across brain regions. It is evident from the bar graphs (Figure 2) and the autoradiographs (Figure 3) that the mean values of rCPS in the two groups (WT and KI\textsubscript{Dutch}) are very similar.

Discussion

The results of the present study show that in a mouse model of FXPM in which the CGG repeat length was between 120 and 159, \textit{in vivo} rCPS measured awake, behaving mice at 4 to 5 months of age were not different from those of WT control mice. Rates were normal despite a 50% decrease in brain FMRP levels. Our study suggests that there may be a threshold level of FMRP necessary for the maintenance of normal rates of protein synthesis.

We used the \textit{in vivo} quantitative autoradiographic leucine method (Smith et al., 1988) to quantify rates of protein synthesis in our studies. With this methodology, we can measure steady-state rates of ongoing protein synthesis in conscious functioning mice. Measurements are average rates of leucine incorporation into all tissue proteins; our measurements do not target specific proteins. The relative contribution of an individual protein to the overall rCPS is weighted by both its fractional contribution to total tissue protein and its half-life in the tissue. The fact that we did not find increased rCPS in the KI\textsubscript{Dutch} mice does not exclude the possibility that the synthesis of specific proteins may be affected, either increased or decreased. One of the advantages of the autoradiographic method is that it enables us to measure rCPS in all regions of the brain. The spatial resolution of the autoradiographic method is 50 μm (Smith, 1983). This permits us to distinguish specific nuclei and cell layers, including dendritic-rich and cell body layers. We cannot, however, distinguish specific cell types within a layer.

The absence of FMRP is at the biochemical core of FXS. FMRP is a polyribosome-associated RNA-binding protein, suggesting that it may play a role in regulating translation. In \textit{in vitro} model systems (Laggerbauer et al., 2001; Li et al., 2001), FMRP binds its target mRNAs and negatively regulates their translation. FMRP has been shown to reversibly stall ribosomes specifically on its target mRNAs during elongation (Darnell et al., 2011). FMRP also recruits cytoplasmic FMRP-interacting protein (CYFIP1) to block formation of the eIF4F complex preventing translation initiation (Napoli et al., 2008). FMRP has also been shown to control translation of the FXS gene 

$\textit{F}(Z, 2) = 0.84$, $p = .39$, indicating that translation is not affected by FMRP in the FXS KO mice (Qin et al., 2005). The most affected regions were hippocampus, hypothalamus, thalamus,
Figure 2. Regional rates of cerebral protein synthesis in WT (open bars) and KI<sub>Dutch</sub> (filled bars) mice. Bars represent the means ± SEM for seven mice in each group except for the paraventricular nucleus of the hypothalamus with five WT mice and four KI mice. Data were analyzed by means of RM ANOVA with region as the within-subjects factor and genotype as the between-subjects factor. The interaction between region and genotype, $F(2.7, 18.9) = 1.154, p = .35$, was not statistically significant, and we found no statistically significant main effect of genotype, $F(1, 7) = 0.676, p = .44$. The main effect of region, $F(2.7, 18.9) = 204.86, p < .001$, was statistically significant.

dHi = dorsal hippocampus; dCA1 = dorsal CA1 pyramidal cell layer; dCA2&3 = dorsal CA2&3 pyramidal cell layer; dRad = dorsal stratum radiatum; dDG = dorsal dentate gyrus; vHi = ventral hippocampus; vCA1 = ventral CA1 pyramidal cell layer; vCA2&3 = ventral CA2&3 pyramidal cell layer; vRad = ventral stratum radiatum; vDG = ventral dentate gyrus; FrCx = frontal association cortex; MCx = primary motor cortex; PFrCx = medial prefrontal cortex; SmCx = somatosensory cortex; PPCx = posterior parietal cortex; CbCx = cerebellar cortex; Str = striatum; BSt = bed nucleus of the stria terminalis; BLA = basolateral amygdala; Th = thalamus; PVN = paraventricular nucleus of the hypothalamus.

Figure 3. Digitized autoradiographic images color coded for rCPS at the level of dorsal hippocampus: (a and c) WT-C and (b and d) KI<sub>Dutch</sub>. Color bar applies to all four images. Scale bar in (b) (0.5 mm) applies to (a) and (b), and scale bar in (d) (1 mm) applies to (c) and (d). Images in (a) and (b) are enlarged subsections through the hippocampus taken from images in (c) and (d), respectively. Lines in (b) delineate the CA1 and the CA2-3 sectors of the pyramidal cell layer of the hippocampus. rCPS = regional rates of cerebral protein synthesis.
amygdala, and frontal and parietal cortex. Studies in hippocampal slices from Fmr1 KO mice confirm increased incorporation rates (Dölen et al., 2007). This core pathophysiological change in the disease might be used as a biomarker of effective drug treatment (Liu et al., 2012; Michalon et al., 2012).

We were interested in knowing whether changes in rCPS occur in FXPM because of reports that patients with FXPM have symptoms suggesting a milder form of FXS (Cornish et al., 2005; Hunter et al., 2008; Kogan et al., 2008; Cornish et al., 2009) and because FMRP concentrations in lymphocytes may be decreased in these patients (Tassone et al., 2000). We hypothesized that with reduced concentrations of FMRP, rCPS might be increased but to a lesser extent than seen in Fmr1 KO mice. In our previous study of the KI NIH mouse model of FXPM with CGG repeat lengths between 120 and 140, we found that FMRP levels in brain were reduced by 80% to 90% of WT and rCPS were increased (106–120% of WT) similar to changes we had seen in Fmr1 KO mice. KI NIH animals also had a behavioral phenotype comparable to Fmr1 KO mice. In the KI Dutch mouse model, we selected mice with CGG repeat lengths between 120 and 159 because animals with repeat lengths in this range had FMRP levels about 50% of WT. The effect that we found on FMRP levels was similar to previous reports in this model. In KI Dutch mice with CGG repeat lengths between 120 and 159, Fmr1 mRNA expression was increased in brain by approximately 1.4 fold compared with WT mice (p <.05), whereas brain levels of FMRP were reduced by 28% (Berman et al., 2012).

Controlling for CGG repeat length, background strain, and age of the animals, the two FXPM mouse models, KI Dutch and KI NIH, yield similar results with respect to changes in Fmr1 mRNA levels and changes in dendritic structure, but with respect to rCPS and behavioral phenotype, the two models diverge. In the KI Dutch model, Fmr1 mRNA levels in brain were increased threefold over WT (Willemsen et al., 2003). Increases were similar in the KI NIH model, two- to fourfold in most brain regions (Qin et al., 2011). Compared with WT mice, KI Dutch mice showed fewer dendritic branches proximal to the soma, reduced total dendritic length, and a higher frequency of longer dendritic spines (Berman et al., 2012). KI NIH mice showed reduced dendritic complexity and increased dendritic spine length in pyramidal neurons in medial frontal cortex, hippocampus, and basal amygdala (Qin et al., 2011). In the present study, we did not determine the behavioral phenotype of KI Dutch animals. Others have shown that KI Dutch mice (CGG repeat length 106–123) are not impaired on the Morris water maze, passive avoidance test, open field test, and tests of neuromotor performance at 140 days of age (Van Dam et al., 2005), although there are some deficits on the Morris water maze and open field tests that occur as the animals age. Further, tests of KI Dutch mice with CGG repeat lengths between 80 and 180 (Hunsaker et al., 2009) indicate some subtle deficits in spatial information processing at 84 and 168 days of age that become more extensive in older animals. Young adult KI NIH mice had a very similar behavioral phenotype to Fmr1 KO mice (Qin et al., 2011), with hyperactivity, reduced anxiety, impaired social interactions, and deficits on passive avoidance test of learning and memory.

The reason for decreased levels of FMRP in the presence of excess Fmr1 mRNA is not understood, but it is thought that transcripts with expanded repeats may impede the linear 40S migration along the 5′-untranslated region (Feng et al., 1995). The similarities in the expanded repeat sequences and Fmr1 mRNA levels in the two models, however, cannot explain the difference in FMRP levels. Moreover, in the generation of both models, the neo cassette was removed by crossing KI mice with mice expressing Cre recombinase, so the changes to Fmr1 were minimal in both models. It has recently been reported that the KI NIH model, but not the KI Dutch model, retains a stop codon 18 bp before the CGG repeat (Todd et al., 2013). Consistent with this difference, FMRpolyG is produced in KI Dutch mice likely through a repeat-associated non-AUG-initiated (RAN) translation mechanism. How the synthesis of FMRpolyG might positively influence FMRP levels is not clear. One proposal is that translation through the repeat may facilitate Fmr1 translation by assisting RNA unwinding by means of helicase recruitment (Todd et al., 2013). With CGG repeat lengths in the normal range, the polyglycine peptides produced in this process may be readily cleared from cells, but expanded repeats (50–200 CGG) produce larger polyglycine proteins that with time accumulate in the cell and are cytotoxic. The decreased levels of FMRP and the absence of FMRpolyG in the KI NIH may suggest that the RAN translation mechanism is important for translation of Fmr1 in the presence of an expanded CGG repeat sequence.

The present and previous studies of the KI Dutch model (Brouwer et al., 2008a; Berman et al., 2012) are in accord with each other with respect to FMRP levels in brains. Our current study suggests that FMRP at 50% of WT levels is sufficient for normal cerebral protein synthesis rates and what appears to be normal brain function in young adult mice.

Conclusions

Our study of the adult male KI Dutch mouse model of FXPM with 120 and 159 CGG repeats showed that in vivo rCPS were normal despite a 50% decrease in
brain FMRP levels. These results suggest that there may be a threshold level of FMRP necessary for the maintenance of normal rates of protein synthesis in the nervous system. As animal’s age and FMRP levels in brain decline, further symptoms may unfold.

**Summary**

In a fragile X premutation mouse model, regional rates of cerebral protein synthesis rates (rCPS) were normal despite 50% fragile X mental retardation protein (FMRP) reductions. Results suggest a threshold level of FMRP may be necessary for maintenance of rCPS. With aging and reduced FMRP, symptoms may unfold.

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