



## Young adult female fragile X premutation carriers show age- and genetically-modulated cognitive impairments

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### ABSTRACT

The high frequency of the fragile X premutation in the general population and its emerging neurocognitive implications highlight the need to investigate the effects of the premutation on lifespan cognitive development. Until recently, cognitive function in fragile X premutation carriers (fXPCs) was presumed to be unaffected by the mutation. Here we show that young adult female fXPCs show subtle, yet significant, age- and *FMR1* gene mutation-modulated cognitive impairments as tested by a quantitative magnitude comparison task. Our results begin to define the neurocognitive endophenotype associated with the premutation in adults, who are at risk for developing a neurodegenerative disorder associated with the fragile X premutation. Results from the present study may potentially be applied toward the design of early interventions wherein we might be able to target premutation carriers most at risk for degeneration for preventive treatment.

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### 1. Introduction

The fragile X premutation is defined by the presence of a CGG trinucleotide repeat expansion between ~55 and 200 in the 5' untranslated region of the fragile X mental retardation 1 (*FMR1*) gene. Alleles in this CGG range produce a 3–8-fold increase in *FMR1* mRNA levels in leukocytes and a decrease in *FMR1* protein (FMRP) levels due to translational inefficiency of the mutant *FMR1* mRNA (Tassone et al., 2000). The full mutation, present in individuals having more than 200 CGG repeats, typically involves methylation, subsequent transcriptional silencing of the *FMR1* gene, and lack of the *FMR1* protein, FMRP (Fu et al., 1991; Pieretti et al., 1991; Snow et al., 1993; Verkerk et al., 1991; Yu et al., 1991). The transcriptional silencing of the gene and the subsequent diminished or absent production of FMRP represents the biological basis for fragile X syndrome (FXS), which results in intellectual disability (Hagerman & Hagerman, 2004), some aspects of which are now evident even in infancy (Farzin & Rivera, 2010).

In the general population, it is estimated that 1 in 260–813 males and 1 in 113–259 females are fragile X premutation carriers (fXPCs; Hagerman, 2008). The fragile X premutation has important implications for multiple members of the same family. For example, a male fXPC will pass the premutation to all of his daughters. His daughters, depending upon their CGG repeat size, will have up to 50% risk of having a child with FXS (Bourgeois et al., 2009). Recent studies suggest that the premutation alleles are associated with several psychiatric and clinical manifestations (Bourgeois et al., 2009), with primary ovarian insufficiency, referred to as fragile X associated primary ovarian insufficiency (FXPOI) affecting 20–28% of women with an *FMR1* premutation (FXPOI; Sullivan et al., 2005) and with the Fragile X Associated Tremor Ataxia Syndrome (FXTAS). FXTAS is a late-onset (>50 years old) neurodegenerative disorder that affects nearly 40% of male and 8% of female fXPCs (Jacquemont et al., 2004) and is associated with intention tremors, cerebellar gait ataxia, parkinsonism, and short term memory and executive function impairments (Bourgeois et al., 2009).

The high frequency of the fragile X premutation in the general population and its emerging neurocognitive implications highlight the necessity of investigating the effects of the premutation on cognitive function, especially during adult development. There are relatively few studies conducted to date, especially in those

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individuals who are asymptomatic for FXTAS. Until recently, cognitive function in fXPCs was presumed to be largely unaffected by the mutation. However, in recent years a number of studies have described significant cognitive impairments in fXPCs across various domains, including working memory and executive function (Cornish et al., 2008; Loesch et al., 2002, 2003; Moore et al., 2004a), memory (Moore et al., 2004b), and arithmetic (Lachiewicz, Dawson, Spiridigliozzi, & McConkie-Rosell, 2006). Cornish et al. (2008, 2009) reported that the working memory and executive functioning impairments were modulated by age and CGG repeat expansion in males with and without FXTAS.

The purpose of the current paper is to report early findings from a large-scale investigation of the neurogenetic mechanisms of cognitive dysfunction in those affected by *FMR1* mutations. Specifically, we focus here on the domain of magnitude comparison. This is one of the most basic aspects of quantitative ability and is thought to develop from non-symbolic, including spatial, representations (Ansari, 2008; Simon, 1997, 1999). Since quantitative and numerical impairments are highly characteristic of those with FXS, one of the key motivations of our studies was to determine whether some of its underlying functions show dysfunction in premutation carriers. A recent finding of poor arithmetic abilities in women who carry the premutation further supports this hypothesis (Lachiewicz et al., 2006). Furthermore, Simon (2007, 2008) has shown that many neurogenetic disorders like FXS, Turner syndrome, Williams syndrome, and chromosome 22q11.2 deletion syndrome have overlapping cognitive dysfunction across the spatial and temporal domains, which creates a suboptimal foundation for the subsequent development of numerical and mathematical competence.

Importantly, impairments in visual, spatial and temporal functions often associated with the dorsal visual processing pathway, which implicates parietal function so frequently associated with numerical and other comparative processes (Cohen Kadosh et al., 2005), have been reported in both adolescent and adult males with FXS and in infants and toddlers with FXS (Kogan et al., 2004; Kéri & Benedek, 2009, 2010). Kogan et al., (2004) conducted experiments to evaluate global motion and form perception mediated by the dorsal and ventral visual streams, respectively. The ventral stream to the temporal lobe receives input from the parvocellular (P) pathway of the lateral geniculate nucleus (LGN); the dorsal stream to the parietal lobe, on the other hand, receives input from the magnocellular (M) pathway of the LGN (Milner & Goodale, 1995). Males with FXS had impaired performance on a global motion task, but had no impairments on a form perception task. Farzin, Whitney, Hagerman, and Rivera (2008) used psychophysical measurements to test first-order (luminance-defined) and second-order (texture-defined) motion and static stimuli in infants with FXS and demonstrated that detection thresholds for only the second-order, dynamic stimuli were significantly elevated in the FXS group. The Farzin et al. results suggest that instead of a general dorsal stream deficit in FXS, abnormalities may exist in higher-level cortical areas responsible for attention-based temporal processing involved in position tracking. More recently, Kéri and Benedek (2009) reported that female fXPCs have impaired M and relatively

spared P visual pathway functioning, suggesting, like Kogan, that spatial and temporal processing is impaired but object and color information processing is not. Kéri and Benedek's (2010) finding that young adult female fXPCs had lower sensitivities for biological (e.g., the movement of humans and animals) and mechanical (e.g., the movement of vehicles and tools) motion compared to non-carriers further confirms a selective spatiotemporal impairment.

In the current study, we assessed simple magnitude comparison ability in asymptomatic (i.e., non-FXTAS), young adult female fXPCs. We used a "numerical distance effect" task (Simon et al., 2008). The task is so named because of the phenomenon of increased response time or difficulty in a relative magnitude judgment as the difference (or numerical distance) between the two quantities decreases (Moyer & Landauer, 1967). We were interested in determining if magnitude comparison impairments existed in young adult female fXPCs since they should be the least affected subpopulation. Like Cornish et al. (2008, 2009), we also wanted to investigate whether such impairments, if they existed, were associated with molecular measures including CGG repeat length, *FMR1* mRNA levels, and age.

## 2. Materials and methods

### 2.1. Participants

Participants were 39 females aged 21–42, including 15 neurotypical (NT) controls and 24 fragile X premutation carriers (fXPCs). The mean age ( $\pm$ SD) for fXPCs was  $32.25 \pm 7.28$  years and for NT controls was  $33.68 \pm 4.42$  years. The two groups did not differ in age,  $t = .68$ ,  $p = .50$ , or Full-Scale IQ,  $t = 2.01$ ,  $p = 0.06$  (see Table 1). Participants were recruited through the NeuroTherapeutics Research Institute (NTRI) at the Medical Investigation of Neurodevelopmental Disorders (M.I.N.D.) Institute of the University of California, Davis Medical Center. This study was approved by the University of California Davis Institutional Review Board and conformed to institutional and federal guidelines for the protection of human participants. Written informed consent was obtained before behavioral testing from all participants.

### 2.2. Psychological assessment

Cognitive ability was based on Full-Scale IQ (FSIQ) using either the Wechsler Adult Intelligence Scale, third edition (WASI-III; Wechsler, 1997) or the Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler, 1999). IQ data were available for 12/15 NT controls and 21/24 fXPCs.

### 2.3. Molecular analysis

#### 2.3.1. CGG repeat size

Genomic DNA was isolated from peripheral blood leukocytes using standard methods (Puregene Kit; Gentra Inc., Valencia, CA). Repeat size was determined using Southern blot analysis as well as PCR amplification of genomic DNA as described in Tassone,

**Table 1**  
Participant descriptive statistics and *FMR1* measures.

	Neurotypical control				Fragile X premutation carrier				<i>t</i>	<i>p</i> -Value
	Mean	SD	Range	<i>n</i>	Mean	SD	Range	<i>n</i>		
Age	32.25	7.28	21–41	15	33.68	4.42	23–42	24	0.68	0.50
Full-Scale IQ	108.23	11.48	89–125	12	116.9	12.12	101–144	21	2.01	0.06
CGG repeat size	30.20	1.22	28–32	10	96.00	21.70	67–143	24	-14.80	<0.0001
<i>FMR1</i> mRNA level	1.61	0.27	1.25–1.98	8	2.3	0.48	1.55–3.42	24	-3.85	<0.0001
Activation Ratio	n/a	n/a	n/a	0	56%	15%	23–79%	24	n/a	n/a

Pan, Amiri, Taylor, and Hagerman (2008). The activation ratio (AR), indicating the percent of cells that carry the normal allele on the active X chromosome, was calculated from the Southern blot as described in Tassone et al. (1999).

### 2.3.2. *FMR1* mRNA

All quantifications of *FMR1* mRNA were performed using a 7900 Sequence detector (PE Biosystems) as previously described (Tassone et al., 2000).

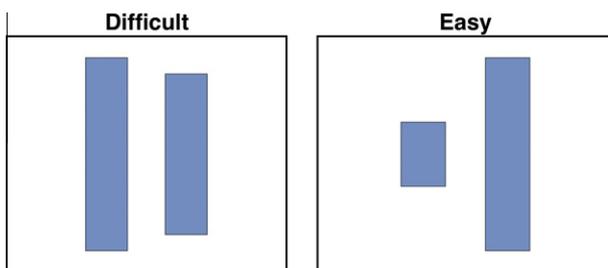
### 2.4. Magnitude comparison (distance effect) task

Participants were asked to indicate which of the two blue bars was larger. To begin each trial, the participant looked at the fixation point on the computer monitor 60-cm away. Once the participant was ready, the stimuli were presented. The bars were vertically oriented, horizontally offset from fixation by 3-cm, and centered at the level of fixation. Each bar was 2-cm wide and varied in height from 1- to 12-cm in 1-cm increments. Example stimuli are presented in Fig. 1. Participants pressed one of two buttons as quickly and accurately as possible to indicate which stimulus was larger. Stimuli were presented until the participant responded or until 7 s had elapsed. Response time and accuracy were recorded as the dependent variables.

There were a total of 120 trials divided evenly into two 60-trial blocks. There was a short rest period between the two blocks of trials. The 60 trials consisted of 10 trials at each of the six possible differences between the heights of the two bars (1, 2, 3, 5, 6, and 7 cm). To reduce the overall number of trials, no height difference of 4 was included in the experiment. We reasoned that since height differences of 4 cm were in the center of our range, they would not contribute much to our understanding of how small and large height differences affected visuospatial cognition. The presentation of the six height differences was counterbalanced within blocks of six trials so that the possible height differences were evenly distributed across the 60 trials pseudorandomly.

### 2.5. Data analysis

Data from the distance effect task measured magnitude comparison as assessed by response time (in ms) and accuracy. These data were blocked according to the six possible height differences of 1, 2, 3, 5, 6, and 7 cm. As in our previous studies (Simon et al., 2008), anticipatory responses and outliers were excluded from the analyses. Anticipatory responses were determined to be any response equal to or less than 150 ms. Outliers were determined as a response greater than or less than three times the interquartile range of the response times at a specific height difference (e.g. 1, 2, 3, 5, 6, 7). After excluding trials with outlier responses, the median reaction time was calculated for each trial condition. Reaction times were



**Fig. 1.** Example stimuli for the numerical distance effect task. On each trial, the participant was asked to indicate, by pressing one of two buttons, which was the larger of the two blue bars. Response time and error rate were used to assess performance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adjusted to reflect both speed and accuracy of performance by combining the median reaction time and error rates using the formula: Median reaction time/(1 – % Error). Using this adjustment, reaction times remain unchanged with 100% accuracy.

One-way analysis of variance (ANOVA) and repeated measures ANOVA were used to assess differences between the groups. To determine the range of a reliable “distance effect”, repeated measures ANOVA models were fit to the data within a group in a sequential manner starting with distances from 1 to 3 cm and adding the next largest distance until a quadratic trend for distance was identified. Once the “distance effect” was identified, analyses focused on those distances. For each individual participant, a simple linear regression model with adjusted reaction time as the outcome and distance as the independent variable was fit to get an estimate of the participant-specific intercept (estimated intercept) and slope (estimated coefficient of distance). These values for each participant correspond to the estimated adjusted reaction time at a distance of 0-cm and how quickly the reaction times drop as the distance increases. These values were then compared between the groups with a one-way ANOVA. Correlations between outcomes and age (for both groups) and molecular variables (FXPCs only) were computed within groups.

Degrees of freedom were adjusted using the Welch procedure for one-way ANOVAs when the equality of variance assumption was violated. For repeated measures ANOVAs, Greenhouse–Geisser corrections were used to correct for violations of the sphericity assumption. Assumptions for all models were checked and were met by the data. Analyses were conducted using SPSS and a  $p$ -value < 0.05 was considered statistically significant.

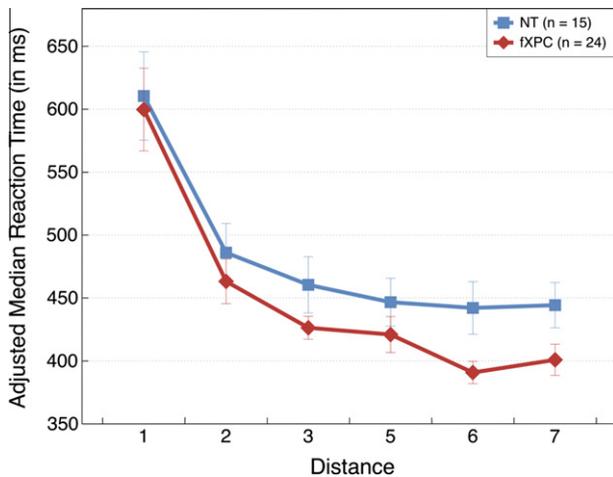
## 3. Results

### 3.1. Molecular analyses

Molecular data were available for 10/15 NT controls and 24/24 fXPCs. Descriptive statistics of CGG repeat size, *FMR1* mRNA, and activation ratios are reported in Table 1. Within the fXPCs, as expected, *FMR1* mRNA level was positively associated with CGG repeat size, Pearson's  $r = .36$ ,  $p$  (one-tailed) = .04, and negatively associated with activation ratio, Pearson's  $r = -.40$ ,  $p$  (one-tailed) = .03. The partial correlation between CGG and *FMR1* mRNA after accounting for the activation ratio in the fXPCs was much stronger, Pearson's  $r = .56$ ,  $p$  (two-tailed) = .005. Prior to examining correlations between *FMR1* molecular variables and cognitive performance, we examined potential confounding effects of age and IQ by computing Pearson correlations between each of these measures and *FMR1* molecular variables. In the female fXPCs, we found no significant correlations between CGG repeat size or *FMR1* mRNA with age or IQ.

### 3.2. Magnitude comparison (distance effect) task

Responses from four female fXPCs for several of the distances in the task were identified as outliers, so they were removed from the analyses that considered all distances. There was no difference in the error rates between the two groups,  $F(1, 33) = .27$ ,  $p > .37$ . Using a repeated measures ANOVA, there was a small significant difference in adjusted reaction times between the two groups,  $F(1, 33) = 4.23$ ,  $p = .05$ , but, surprisingly, it was the female fXPCs who, as a group, responded faster, on average, than female NT controls (see Fig. 2). Adjusted reaction times increased as the difference between the two blocks decreased,  $F(5, 165) = 62.39$ ,  $p = .0001$  and did not differ between the groups,  $F(5, 165) = .32$ ,  $p = .72$ . The observed error rates were extremely small (only 2% of the observations across all participants and distances had errors), so the adjusted times were very close to the observed



**Fig. 2.** Group analyses of adjusted reaction times show that female fXPCs, as a whole, responded faster than female NT controls,  $p = .05$ . Adjusted reaction times increased as the difference between the two blocks decreased,  $p = .0001$  and did not differ between the groups,  $p = .72$ . Error bars represent standard error of the mean.

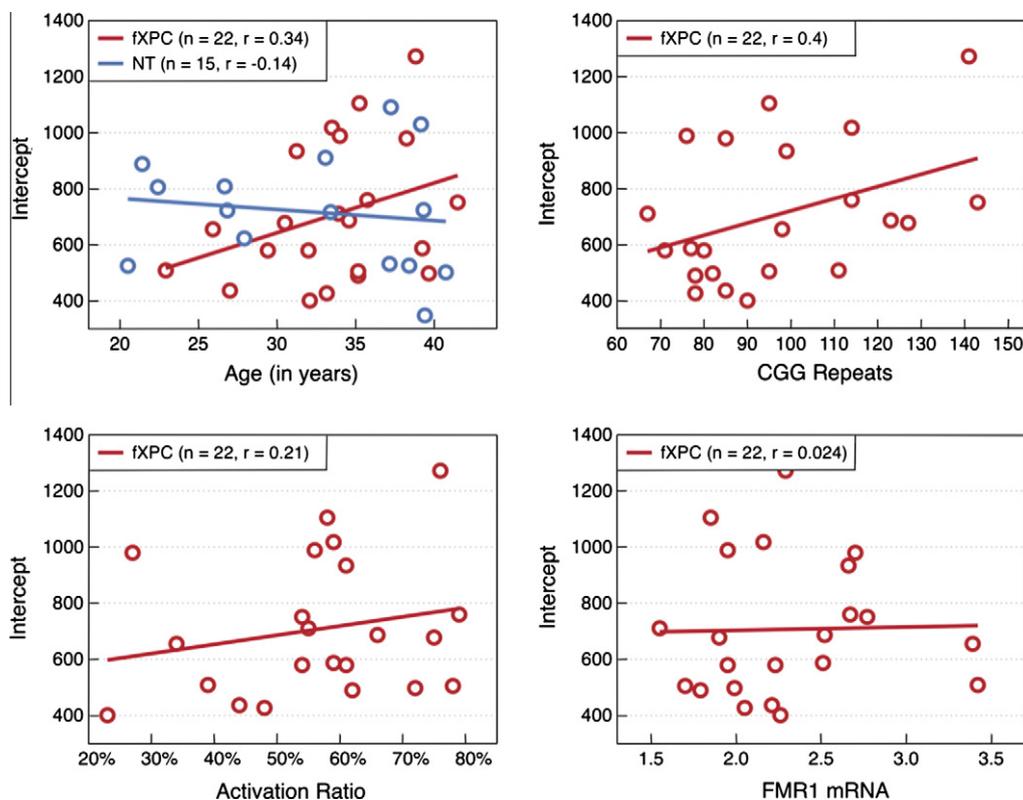
reaction times. However, we reran the analyses using the unadjusted times and found that the results were similar.

Analyses to identify a reliable “distance effect” were performed in each group separately. For the NT controls, a significant quadratic trend emerged with 1- to 3-cm, indicating a “distance effect” range from 1- to 2-cm,  $F(1, 14) = 8.50$ ,  $p = .01$ . For the female fXPC group, a significant quadratic trend emerged with 1- to 3-cm, indicating a “distance effect” range from 1- to 2-cm as well,  $F(1, 19) = 24.11$ ,  $p < .0001$ . Because quadratic trends were identified over the range

of 1- to 3-cm for both groups, we focused on distances from 1- to 2-cm, where the changes in adjusted reaction times were linear for the remaining comparisons.

Intercepts and slopes for the linear fit lines through the points at distances 1- and 2-cm were estimated for each person. Two of the individuals removed from the earlier analyses were included in the following analyses, because they did not have any outliers identified for distances of 1- or 2-cm. Results were similar if they were removed from the analyses. The average intercept was  $707.19 \pm 244.05$  ms for female fXPCs and  $717.08 \pm 210.41$  ms for NT controls. The average slope was  $-125.48 \pm 92.89$  ms/distance for fXPCs and  $-111.74 \pm 93.63$  ms/distance for NT controls. Intercepts and slopes were not different between the groups,  $p > .6$ , for both.

Further investigation into the intercepts and slopes within each group assessed the association between these measures and age (both groups) and molecular variables (in the female fXPCs). There were two positive associations for female fXPCs; one between age and intercept, Pearson's  $r(20) = .34$ ,  $p$  (one-tailed) = .06 and one between CGG repeat length and intercept, Pearson's  $r(20) = .40$ ,  $p$  (one-tailed) = .03. Slopes were not significantly associated with any of the variables within the fXPCs. There were no significant associations between intercept or slope and age for NT controls. Fig. 3 presents the observed associations between age and intercept for each group. There was no significant difference in the association between intercept and age by group,  $p = .12$ . We confirmed these correlations by also computing the partial correlation accounting for the activation ratio. The partial correlation between CGG and intercept after accounting for the activation ratio in the fXPCs was still significant, Pearson's  $r = .36$ ,  $p$  (one-tailed) = .05. No other partial correlations were significant between molecular variables and slope or intercept.



**Fig. 3.** Positive associations were observed for female fXPCs; one between age and intercept, which approached significance,  $p$  (one-tailed) = .06 and one between CGG repeat length and intercept,  $p$  (one-tailed) = .03. Slopes were not significantly associated with any of the variables within the fXPCs. No significant associations were observed between intercept or slope and age for NT controls. There was no significant difference in the association between intercept and age by group,  $p = .12$ .

#### 4. Discussion

In the current study, we sought to quantify simple magnitude comparison ability in asymptomatic (i.e., non-FXTAS), young adult female fragile X premutation carriers (fXPCs). In addition, we sought to explore the effects of age, CGG repeat length, and *FMR1* mRNA levels with performance.

When we compared female fXPCs to neurotypical (NT) control participants, our results appeared to be consistent with those of many previous studies that detected no cognitive impairments in participants carrying the fragile X premutation. As a group, the female fXPCs produced similar magnitude judgments and were faster to respond than the neurotypical adults on the magnitude comparison task.

However, our in-depth analyses beyond the main effects revealed several findings that are remarkable in that they do not fit the currently accepted view of the unimpaired cognitive status of young adult female premutation carriers. Specifically, our data indicate that female fXPCs show evidence of subtle, yet statistically significant, degradations in cognitive function as measured by a magnitude comparison task that are related both to age and to "gene dosage" of the *FMR1* gene mutation.

In the fXPC group, we found a pattern in which magnitude comparison performance deteriorated as the age of the participants increased from 20 to 42 years of age. This relationship was almost strong enough to reach significance at the alpha level of  $p < .05$ . There was no such relationship in the group of NT participants, as can be seen in Fig. 3. This finding does not necessarily indicate any evidence of neurocognitive degeneration in the group of women carrying the fragile X premutation. Indeed, inspection of Fig. 3 shows little difference in terms of the spread of intercept values in the older women in either group, indicating that advancing age did not introduce more impaired performance. Instead, it is evident from the figure that there are more young women in the fXPC group than in the NT group who produced rather small intercept values associated with better performance on the task. Why this pattern exists, if indeed it is replicated, is not presently clear and it clearly requires further study.

An even stronger relationship was found in the fXPC group when we investigated how intercept values varied with CGG repeat length. When "dosage" was measured in terms of the CGG repeat length in the fXPC group there was a significant positive relationship indicating that performance was significantly poorer as repeat length increased from 67 toward 150. Again, the reason for this relationship is not immediately clear and replication is obviously required. However, a clear suggestion is that the reduction in FMRP that is assumed to increase as CGG length increases is in some way responsible for this apparent impairment. How and when, during the lifespan, this takes place and how it might be related to the impact of gene silencing in the full mutation of *FMR1* gene also needs further extensive investigation.

Interestingly the effect was not evident when performance was related to leukocyte *FMR1* mRNA levels. This last observation is not too surprising given that leukocyte *FMR1* mRNA levels is not an accurate measure of *FMR1* mRNA levels in the brain. The premutation allele result in a 3–8-fold increase in leukocyte and only a 1–2-fold increase in brain *FMR1* mRNA levels (Tassone et al., 2000). Brain levels of *FMR1* mRNA also vary widely across different brain regions (Tassone, Iwahashi, & Hagerman, 2004). Other studies have also reported impairments correlated with CGG repeat expansion but not leukocyte *FMR1* mRNA levels, which corroborate our results (Greco et al., 2006; Leehey et al., 2008).

Clearly, some limitations exist in these very early investigations of the developmental neurocognitive endophenotype of young adults carrying fragile X premutations. First of all, our study was

cross-sectional in nature and so it should be stressed that our data do not allow us to determine at this point if the age effect is a stable phenotype or a subtle precursor of a late-onset neurodegenerative process. Further studies, especially longitudinal studies, might aid in identifying if this result is stable or not and whether other environmental factors contribute to early degeneration. We are also unable to predict what type of aging function may exist (e.g., linear, exponential, etc.) and future directions will consist of testing a greater number of participants within the current age range of 20–40 year olds as well as testing older participants.

Further, although performance was positively associated with CGG repeat length in the fXPC group, we recognize that this may not be the most reliable marker of molecular pathology. As in previous reports, as CGG repeat length expanded in the current fXPC group, *FMR1* mRNA levels slightly increased. Elevated *FMR1* mRNA is thought to produce a toxic gain of function by which proteins bind excessively to the expanded CGG repeat and are then sequestered (Garcia-Arocena & Hagerman, 2010). However, without FMRP levels, the outcomes of elevated *FMR1* mRNA are unknown. FMRP has not yet been quantified in the participants, so unfortunately no direct correlation can be drawn between markers of molecular pathology and magnitude comparison impairments. Studies are currently underway to quantify FMRP levels in these participants using a recently published enzyme-linked immunosorbent assay (ELISA) method (Iwahashi et al., 2009).

Our original hypothesis was that asymptomatic, young adult female fXPCs would not be grossly cognitively impaired compared to female NT controls. There remained the possibility that subtler cognitive dysfunction might exist, and that this may be a consequence of age and variables of the *FMR1* gene but this was not our initial prediction. These novel and surprising results add to a growing body of evidence that suggests the premutation allele is associated with neurocognitive dysfunction and that this may have a much longer developmental trajectory than previously thought. Our data cannot yet determine if our findings represent the earliest indication of a "risk prodrome" for FXTAS or a more stable attenuated phenotype that requires other influences to move toward the degenerative disease. In either case, the implications for early detection and prevention of disease are significant and further studies may be able to identify targets for potential neurotherapeutic interventions.

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