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Investigation of amygdala volume in men with the fragile X premutation

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Abstract

Premutation fragile X carriers have a CGG repeat expansion (55 to 200 repeats) in the promoter region of the *fragile X mental retardation 1 (FMR1)* gene. Amygdala dysfunction has been observed in premutation symptomatology, and recent research has suggested the amygdala as an area susceptible to the molecular effects of the premutation. The current study utilizes structural magnetic resonance imaging (MRI) to examine the relationship between amygdala volume, CGG expansion size, *FMR1* mRNA, and psychological symptoms in male premutation carriers without FXTAS compared with age and IQ matched controls. No significant between group differences in amygdala volume were found. However, a significant negative correlation between amygdala volume and CGG was found in the lower range of CGG repeat expansions, but not in the higher range of CGG repeat expansions.

Keywords

Fragile X premutation; Amygdala; *FMR1*; mRNA; Structural MRI

Introduction

Premutation fragile X carriers have a CGG repeat expansion (55 to 200 repeats) in the promoter region of the fragile X mental retardation 1 (*FMR1*) gene and are at greater risk for social, emotional, and cognitive deficits, including autism spectrum disorders (ASD) (Borghgraef et al. 2004; Cornish et al. 2005; Dorn et al. 1994; Farzin et al. 2006; Franke et al. 1998; Hagerman and Hagerman 2002; Johnston et al. 2001; Moore et al. 2004a, b; Tassone et al. 2004b). Female premutation carriers have a higher rate of premature ovarian insufficiency (Allingham-Hawkins et al. 1999; Schwartz et al. 1994; Sherman 2000) and psychiatric problems (Bourgeois et al. 2009; Roberts et al. 2009), and their repeat region often expands between generations so that their children frequently inherit full-mutation forms (CGG repeat expansions to >200 CGG repeats) of the *FMR1* gene (Entezam et al. 2007), which gives rise to fragile X syndrome. Premutation males, and to a lesser extent females, often develop a late onset neurodegenerative disorder known as fragile X-associated tremor/ataxia syndrome (FXTAS) (Adams et al. 2007; Hagerman and Hagerman 2004b; Hagerman et al. 2001; Jacquemont et al. 2003). FXTAS is associated with cerebellum, cerebrum, and whole brain volume loss (Cohen et al. 2006), diffuse grey-matter density loss, especially in the cerebellum and limbic regions (Hashimoto et al. 2011b), as well as both white-matter lesions and other abnormalities in the middle cerebellar peduncles (Hashimoto et al. 2011a). Other characteristics of those with FXTAS include memory and executive function impairments, cognitive decline, parkinsonism, peripheral neuropathy, and autonomic dysfunction (Bacalman et al. 2006; Brunberg et al. 2002; Grigsby et al. 2006, 2007; Jacquemont et al. 2004). Numerous studies have also found differences in behavioral and psychiatric measures as well as brain function in younger premutation carriers who do not display any overt neurological symptoms (Cornish et al. 2005; Farzin et al. 2006; Hessler et al. 2005, 2007; Koldewyn et al. 2008). Additionally, two recent studies have found both grey matter density differences (Hashimoto et al. 2011b) and FA, axial and radial diffusivity differences in regions of the cerebellum in younger male premutation carriers without FXTAS (Hashimoto et al. 2011a). It is therefore important to examine both molecular and neurological factors that could contribute to symptomatology and changes in brain structure and function in younger premutation carriers without FXTAS. Doing so will not only elucidate the factors affecting premutation carriers across the life-span, but allow us to examine how these factors may relate to later FXTAS development.

On the molecular level, the premutation results in increased *FMR1* mRNA across the premutation range, although *FMR1* mRNA is highly correlated with CGG repeat number so that those with large CGG expansions are likely to have both the highest levels of *FMR1* mRNA and some reduction in FMRP levels (Allen et al. 2004; Kenneson et al. 2001; Tassone et al. 2000a, b), the latter likely due to a deficit in translational efficiency (Primerano et al. 2002). FMRP, an RNA binding protein, regulates the translation of many gene products and has been implicated in neuronal growth and development (Inoue et al. 2000; Khandjian 1999). Lack of FMRP due to silencing of the *FMR1* gene is the cause of intellectual disability in individuals with fragile X syndrome (Feng et al. 1995; Verkerk et al. 1991; Yu et al. 1991), and even small decrements in FMRP could potentially lead to significant neuronal consequences (Feng et al. 1995; Kenneson et al. 2001; Tassone et al. 1999). Our current hypothesis is that elevated *FMR1* mRNA causes a toxic gain of function effect in those with the premutation (Amiri et al. 2008; Brouwer et al. 2009; Hagerman and Hagerman 2004a) and this, combined with changes in FMRP production, may be responsible for many of the cognitive, psychiatric, and neurological characteristics of the premutation phenotype.

The memory (Jäkälä et al. 1997; Moore et al. 2004a), emotion (Cornish et al. 2005), and psychological symptoms (Hessl et al. 2005; Roberts et al. 2009) associated with the premutation have implicated the limbic region as a possible origin of some premutation symptomatology. Although several previous studies have looked at hippocampal structure and function (Abitbol et al. 1993; Adams et al. 2010; Greco et al. 2002; Jäkälä et al. 1997; Koldewyn et al. 2008; Moore et al. 2004b), fewer studies have focused on the amygdala in the premutation population (Hessl et al. 2007; Moore et al. 2004b). During normal fetal development, the nucleus basalis magnocellularis (nBM), an area with extensive cholinergic projections to the amygdala, has high levels of *FMR1* gene expression (Abitbol et al. 1993). This suggests that changes in *FMR1* gene expression observed in premutation carriers, including both mRNA increases and FMRP decreases, could potentially affect the nBM, and result in altered innervation of the amygdala. Such changes in amygdalar innervation early in development could result in altered amygdala structure and function throughout the lifespan. This hypothesis is supported by the observation that *FMR1* mRNA levels are disproportionately increased in the amygdala of premutation carriers (Tassone et al. 2004a, b).

An fMRI study from our lab tested the hypothesis of possible amygdala dysfunction and found male premutation carriers to have less amygdala activation compared to controls while viewing fearful faces compared to scrambled faces (Hessl et al. 2007). Within this group, reduced amygdala activation was negatively correlated with psychological symptoms on the Symptom Checklist-90 Revised (SCL-90-R). Premutation participants compared to controls were also found to have decreased potentiation of the eye blink startle reflex to fearful faces and diminished skin conductance during a brief social stressor (Hessl et al. 2007).

In previous research by Moore et al., the amygdalo-hippocampal complex was one of several brain regions found to have significantly less voxel density among 20 male premutation carriers compared to 20 age and IQ matched controls. In addition, premutation group volumetric results were negatively correlated with age and CGG trinucleotide repeat expansion, and positively correlated with blood lymphocyte FMRP expression (Moore et al. 2004b). While this study did not control for FXTAS, significant differences were seen in younger participants where FXTAS would be very unlikely. Although a subsequent study found the hippocampus to have significantly reduced volumes in premutation carriers compared to controls (Jäkälä et al. 1997), significant volume differences in the amygdala have not yet been demonstrated. A previous study by our group, primarily focused on

assessing amygdala function, did find raw left amygdala volumes to be correlated with *FMRI* mRNA in a small group of men with the premutation; although adjusting for total brain volume reduced the correlation to trend levels. A significant correlation was also demonstrated between adjusted right amygdala volumes and psychological symptoms on the SCL-90-R. Although no overt amygdala volume differences between premutation carriers and age and IQ matched controls were found, the sample size was small ($n=13$ controls, $n=12$ premutations) and the study may have lacked sufficient power to detect true volumetric differences between groups (Hessl et al. 2007).

Based on the prior body of research implicating the amygdala as a region affected by the premutation, the current study used structural magnetic resonance imaging to examine a large data set of male premutation carriers without FXTAS to investigate whether premutation status affects amygdala volume specifically, and how these structural effects might be associated with CGG expansion size, *FMRI* mRNA, and psychological symptomatology. Based on previous research and the theory of mRNA toxicity, we predicted amygdala volumes to be smaller in the premutation group compared to controls, and for amygdala volumes to be negatively correlated with CGG expansions, mRNA, and psychiatric symptomatology within the premutation group.

Materials and methods

Participants

Participants included 49 men (mean age=48.5 years) with a confirmed premutation *FMRI* allele and a comparison group of 48 men (mean age=47.9 years) without the premutation. Males with the premutation were recruited through screening of pedigrees of probands with FXS, and none of the premutation men had children with the full mutation. Only men were included in the current study in order to avoid the confounding effect of X-chromosomal activation ratios in females. The two groups were matched for age ($t(95)=-0.191$, $p=0.849$), and Full Scale IQ (premutation, 116.17; control, 120.49; $t(93)=1.33$, $p=0.187$). Participants' descriptive statistics and *FMRI* data are shown in Table 1. Neurological examination performed by a physician (RJH) ruled out the presence of tremor or ataxia in the premutation carriers. It is important to note that, although she has extensive experience with premutation carriers and with FXTAS, this physician is not a neurologist so it is possible that some participants with very mild symptoms of FXTAS could be included in our sample. Controls were recruited either through the medical center community or were non-carrier males in families affected by fragile X. None of the patients showed any evidence of FXTAS. All participants signed an informed consent approved by the University of California at Davis Institutional Review Board.

Psychological measurement

Intelligence—Cognitive ability was based on full scale IQ using the Wechsler Adult Intelligence Scale, Third Edition (Wechsler 1997).

Psychological symptoms—Psychological symptoms were assessed using the SCL-90-R (Derogatis 1994). The SCL-90 has been extensively used in research paradigms to assess current psychological symptoms, though it is not a standard for clinical diagnostic assessment purposes. The SCL-90-R is a standardized self-report inventory of current psychological symptoms consisting of 90 items, each rated on a five-point scale of distress and clustered into the following symptom dimensions: Somatization, Obsessive–Compulsive, Interpersonal Sensitivity, Depression, Anxiety, Hostility, Phobic Anxiety, Paranoid Ideation, and Psychoticism. The Global Severity Index (GSI) is an indicator of overall level of psychological disturbance within the past week.

Molecular and genetic measures

DNA and mRNA analysis—FMR1 mRNA expression levels were measured with real time quantitative fluorescence RT-PCR method as previously described (Tassone et al. 2000a, b).

Peripheral blood lymphocytes (5 ml of whole blood using standard methods (Puregene Kit; Gentra Inc.) were used to isolate genomic DNA. CGG repeat size was determined using both Southern blot analysis and PCR amplification. Southern blot analysis included digesting 5–10 µg of isolated DNA with EcoRI and NruI and hybridization with the *FMR1* genomic dig-labeled StB12.3 probe. PCR amplification utilized primers c and f (Fu et al. 1991) and a dig-end-labeled oligonucleotide probe (CGG)₁₀ for hybridization. Both analyses were performed using an Alpha Innotech FluorChem 8800 Image Detection System. Further details of the method are described in Tassone et al. 2008.

Brain volume

MRI image acquisition—MRIs were acquired at the UC Davis Imaging Research Center using both a 1.5 T GE Signa Horizon LX NV/I scanner with Echospeed gradients and a standard GE whole head coil and a 3 T Siemens Trio with an eight channel head coil. The T1 weighted spoiled grass gradient recalled (SPGR) 3D MRI sequence acquired with the 1.5 T GE scanner has a 1.3 mm³ resolution, 256×256×124 matrix, Flip angle=15°, and FOV 220 mm. Two separate T1 magnetization prepared rapid acquisition gradient echo (MPRAGE) 3D MRI sequences were acquired with the 3 T Siemens scanner. The first sequence has a 1.0 mm³ resolution, 256×256×192 matrix, Flip angle=7°, and FOV 256 mm. The second sequence has a 0.9 mm³ resolution, 243×234×198 matrix, Flip angle=7°, and FOV 243 mm³.

MRI volumetric analysis—All volumetric measures were completed with the use of Mayo BIR's Analyze 8.5 software (Robb 2001; Robb and Barillot 1989; Robb et al. 1989). Scans in our dataset were of three differing native resolutions: one from the 1.5 T scanner with a resolution of 1.3 mm×0.85 mm×0.85 mm and 2 from the 3 T scanner with resolutions of 0.95 mm×0.475 mm×0.475 mm and 1 mm×1 mm×1 mm. All images for each subject were re-sliced using cubic-spline interpolation to yield uniform voxel dimensions of .5 mm×.5 mm×.5 mm.

Amygdala measurement—For the amygdala volumetric measurements, images were reoriented such that the long-axis of the hippocampus ran parallel to the horizontal axis. The caudal two-thirds of the amygdala were delineated in the coronal view. In the most caudal portions, the amygdala profiles were bounded by the optic tract medially, the substantia innominata along the dorsal portions, white matter along the lateral aspects, and lateral ventricle along the ventral surface. Progressing rostrally, our profiles lay contiguous with the medial surface of the temporal lobe along an edge bounded medially by semiannular sulcus proceeding towards the most lateral aspects of the surface. In the caudal to rostral direction, the dorsolateral aspects of the amygdala profiles were bounded first briefly by the anterior commissure and then by portions of the ventral putamen and ventral claustrum. White matter consistently bounded our tracings along the lateral aspects. Along the ventral edge, outlying borders initially consisted of the surface of the temporal horns of the lateral ventricle and progressed to the alveus of the hippocampus after it abuts the amygdala in the slice cross-section. Progressing rostrally, the ventral boundaries ended just lateral of the entorhinal cortex leading towards the lateral aspects of the uncus, which consisted of the medial body leading into the semiannular sulcus.

After defining approximately the caudal two-thirds of the amygdala, the transverse view was used to further refine the borders with respect to the dorsolateral aspects of the amygdala that abut the putamen in the most caudal regions. The rostral third of the amygdala was then delineated in the sagittal view by extending the ventral aspect up to the white matter ascending from the ventral hippocampus and then curving up towards the dorsal surface of the temporal lobe. Tracing profiles were then reviewed one final time in the coronal plane. The tracing guidelines used in this study have been established in Schumann et al. 2004 and the tracing procedure is illustrated in Fig. 1. All amygdala volumes were traced by a single blinded rater (D.S.) who achieved an intrarater reliability (intraclass correlation coefficient) for the left and right amygdala of 0.91 and 0.92, respectively.

Total cerebral volume measurement—Images were first aligned to place the Anterior Commissure and the Posterior Commissure along a horizontal axis. The images were then re-interpolated to yield 5 mm thick coronal sections and subsequently skull stripped manually. Non-cerebral brain matter such as the brainstem and the cerebellum were also removed. Segmentations were then produced by processing the images through a Gaussian filter classifying brain and non-brain material into two classes. For more information about the protocol used for measuring the total cerebral volume, refer to Schumann et al. 2004.

Total cerebral volume measurements were performed by 2 blinded raters. An interrater reliability (intraclass correlation coefficient) of 0.99 was achieved between raters before volume measurements were performed.

Statistical analysis

Primary outcomes included total cranial volume and corrected left and right amygdala volumes (adjusted for total cranial volume). Two-sample t-tests were used to compare volumes between the groups. Separate linear regression models were then used for each outcome to assess group differences after accounting for age and scanner type. All model assumptions were checked and were met by the data. Within group correlations were assessed between volumes and CGG, mRNA, and psychiatric symptoms. All analyses were performed using the R statistical software, version 2.9.1 (R Development Core Team (2009)) and a p -value < 0.05 was considered statistically significant.

Results

Out of the total 97 subjects, 21 (43.7%) control participants and 25 (51%) premutation carriers were scanned at 1.5 T, with the remaining individuals scanned at 3 T. There was no difference in the percentage scanned at the two field strengths by group ($p=0.61$). Total brain and amygdala volume statistics are shown in Table 2. Independent sample t-tests showed no difference between groups in total cranial volume ($p=0.63$), raw right amygdala volume ($p=0.66$), or raw left amygdala volume ($p=0.34$); adjustment for total cranial volume still did not show differences (right: $p=0.80$, left: $p=0.70$). Linear regression models accounting for age and scanner-type found older age associated with larger amygdala volumes but smaller total cranial volume ($n=97$; corrected right amygdala volume: $t=2.9$, $p=0.005$, corrected left amygdala volume: $t=3.7$, $p<0.001$, total cranial volume: $t=-7.2$, $p<0.001$) and 3 T scanner associated with larger amygdala volumes than the 1.5 T scanner ($n=97$; corrected right amygdala volume: $t=2.5$, $p=0.014$, corrected left amygdala volume: $t=3.5$, $p<0.001$). Despite including both age and scanner-type as covariates, the groups were not significantly different for any of our volumetric measures ($n=97$; corrected right amygdala volume: $t=0.4$, $p=0.69$, corrected left amygdala volume: $t=-0.21$, $p=0.83$, total cranial volume: $t=-0.4$, $p=0.69$) (See Fig. 2). Secondary analyses investigated an interaction between scanner-type and group to see if results were similar by scanner-type. This

interaction was not quite significant for corrected right amygdala volume ($t=-1.9$, $p=0.06$) nor for corrected left amygdala volume ($t=-1.8$, $p=0.07$) with differences between the groups slightly smaller at 3 T than at 1.5 T, and was not significant for total cranial volume ($t=0.1$, $p=0.92$). Contrasts were derived to test for group differences for each scanner-type and found no significant difference at 1.5 T (corrected right amygdala volume: $p=0.10$, corrected left amygdala volume: $p=0.25$) or 3 T (corrected right amygdala volume: $p=0.30$, corrected left amygdala volume: $p=0.16$).

In controls, corrected amygdala volume was not significantly correlated with either CGG ($n=48$; right: *Spearman's rho*=0.015, $p=0.92$, left: *Spearman's rho*=0.123, $p=0.41$) or mRNA ($n=48$; right: *Spearman's rho*=0.191, $p=0.19$, left: *Spearman's rho*=0.097, $p=0.51$). In the entire premutation group, corrected amygdala volumes also did not significantly correlate with CGG ($n=49$; right: *Spearman's rho*=-0.267, $p=0.06$, left: *Spearman's rho*=-0.259, $p=0.07$) or with mRNA ($n=45$; right: *Spearman's rho*=-0.284, $p=0.06$, left: *Spearman's rho*=-0.162, $p=0.29$). As expected, however, CGG and mRNA were highly correlated with each other ($n=45$; *Spearman's rho*=0.843, $p<0.001$) within the premutation group.

As there is mounting evidence that there may be different molecular mechanisms at work in high-repeat premutation carriers and lower-repeat premutation carriers, we split the premutation group into two groups: a low-repeat group (CGG ≤ 55 and <85 , $n=22$) and high-repeat group (CGG ≥ 85 , $n=27$). Low and high repeat group descriptive statistics and *FMR1* measures are shown in Table 3. Twelve (54%) of the low CGG repeat individuals and 13 (48%) of the high CGG repeat individuals were scanned at 1.5 T with the remaining individuals scanned at 3 T. These percentages did not differ by high or low CGG repeat status ($p=0.87$). Partial correlations controlling for age effects on our volumetric data revealed CGG to be significantly negatively correlated with both corrected right amygdala volumes ($n=22$; $r=-0.56$, $p=0.007$) and corrected left amygdala volumes ($n=22$; $r=-0.51$, $p=0.02$) in the low-end premutation carriers but not the high-end group ($n=27$; corrected right amygdala volumes $r=-0.05$, $p=0.80$, corrected left amygdala volumes $r=-0.05$, $p=0.80$). Figure 3 illustrates these findings. Estimated partial correlations were similar when restricted to a particular scanner type for the corrected volumes, but were less consistent for the raw volumes (data not shown). The split point for separating the premutation participants into high- and low-repeat groups was not important to these conclusions. The same relationships were revealed at several different cut-points (80, 90, 100). Splitting the groups at 85 repeats was chosen primarily because it allowed us to separate premutation carriers into two roughly equal groups.

No between group differences were found on the SCL-90-R GSI ($p=0.50$). Additionally, no correlations were found in the premutation group between volumetric variables and SCL-90-R GSI ($n=42$; corrected right amygdala volume: $r=-0.21$, $p=0.17$, corrected left amygdala volume: $r=-0.14$, $p=0.39$, total cranial volume: $r=0.20$, $p=0.21$) or the SCL-90-R ANX ($n=42$; corrected right amygdala volume: $r=-0.10$, $p=0.54$, corrected left amygdala volume: $r=-0.13$, $p=0.40$, total cranial volume: $r=0.16$, $p=0.30$).

Discussion

We examined amygdala structure and its relation to CGG repeat expansion size, *FMR1* mRNA, and psychological symptomatology in both typical controls and non-FXTAS carriers of the fragile X premutation. As a group, premutation carriers compared to IQ and age matched controls showed no significant difference in amygdala size. These results are in contrast to the study by Moore et al. (2004b), which reported significantly decreased voxel density in the amygdalo-hippocampal complex. The difference between our findings and

those of Moore et al. could be explained by a number of factors. First, the methods used to examine volume were quite different: the current study used a rigorous and time-intensive manual segmentation procedure performed within single-subject space while Moore and colleagues utilized voxel-based morphometry techniques within normalized space. Additionally, their findings were reported in the amygdalo-hippocampal complex and could be driven strictly by hippocampus volume decrements, a morphological difference which has been reported in the premutation phenotype by Jäkälä and his colleagues (Jäkälä et al. 1997). Additionally, Moore et al. did not screen for the presence of FXTAS in their older participants, so it is possible that volumetric differences shown in their cohort were influenced by individuals who had or were predisposed to FXTAS. The current sample of premutation carriers included only those whom were confirmed to have no signs of FXTAS at the time of scan, many of whom were of advanced age. Thus, another reason for the discrepancy between the two sets of findings may be that our sample could include relatively more participants for whom unknown protective factors prevent or slow the development of FXTAS symptomatology, and those same factors could be protecting them from amygdala volume loss. Nevertheless, while somewhat unexpected, the current results do confirm that previous amygdala dysfunction exhibited by men with the premutation (Cornish et al. 2005; Hessel et al. 2007) is not being driven by gross volume reduction in this structure.

Our original hypothesis, in line with previous findings of both structural (Moore et al. 2004b) and functional differences in the amygdala (Cornish et al. 2005; Hessel et al. 2007), was that with this substantially larger sample size we would find smaller amygdalae in those with the premutation and that decrements in amygdala volume would be negatively correlated with CGG repeat size and mRNA levels across the premutation range with the largest effects seen in high-repeat carriers. Given that we expected volumetric changes to be related to the molecular phenotype of the premutation, which vary widely across participants, that we did not find large volumetric differences between our two groups as a whole is not necessarily surprising. What was particularly intriguing was our finding that it was the low-repeat group that showed a relationship between CGG and volumetric changes rather than the high-repeat group, although due to small sample sizes, these results should be interpreted with caution.

There are several possible explanations for this potential difference between high-repeat and low-repeat premutation carriers, though all of them are somewhat speculative. In high-repeat carriers, increased levels of *FMRI* mRNA are associated with increased translational difficulty, resulting in decreased levels of FMRP in some high-repeat carriers (Kenneson et al. 2001; Tassone et al. 2000a). Thus, although there is considerable heterogeneity in the degree of increase in *FMRI* mRNA within the high premutation group, most high-repeat carriers are likely to have at least some degree of reduced FMRP production. This expression heterogeneity, as well as the possibility that increased mRNA and decreased FMRP may affect the premutation phenotype in different ways, complicates the molecular picture and may explain why we found no consistent relationship between our molecular measures and amygdala volume in the high-repeat group. Additionally, there is a tighter correlation between CGG repeat number and mRNA in lower-repeat premutation carriers than in high-repeat premutation carriers (Tassone et al. 2000a, 2007). This weakening of the CGG repeat/mRNA correlation may also contribute to our failure to see molecular/volume relationships in the high-repeat group. In premutation carriers with lower repeat numbers, *FMRI* mRNA is already significantly increased (Tassone et al. 2000a, 2007), but may not yet significantly impede translation; in which case increased levels of mRNA might actually result in *increased* levels of FMRP (Peprah et al. 2009). Thus the molecular phenotype in these two groups of premutation carriers may be quite different, obscuring possible group

differences from typical controls and making interpreting research results in the group as a whole quite complex.

When the high-repeat and low-repeat premutation carriers were analyzed as two separate groups, we found a negative correlation between CGG repeat size and bilateral corrected amygdala volume in the low-repeat group. These results may relate to those reported by Moore et al. (2004b), where amygdalo-hippocampal voxel density was found to be negatively correlated with CGG expansion size in the premutation group. In our data set, we also found that *FMR1* mRNA levels were negatively correlated with raw right and left amygdala volumes (data not shown) but this correlation did not survive correction of the amygdala volumes for total cerebral volume. In line with current theories of the molecular mechanism at work in premutation carriers (Hagerman and Hagerman 2004a), we hypothesized that abnormally high levels of *FMR1* mRNA may have a toxic gain-of-function effect in the brain and be a main effector in volume differences or loss seen in those with the premutation. As such, we expected a tighter correlation between volume and mRNA levels than CGG repeat number. It is important to note, however, that *FMR1* mRNA ascertained from blood samples almost certainly does not accurately represent brain mRNA levels. In a study examining post-mortem brain tissue from a single premutation carrier, *FMR1* mRNA expression varied among different brain regions while CGG repeat expansion size was the same among the various tissue types (Tassone et al. 2004a). Furthermore, while the increased expression of *FMR1* mRNA measured in the blood was replicated in most regions of the brain, the amount of increase over normal levels was substantially less in brain than in blood, suggesting that there is tighter control of *FMR1* gene expression in brain tissue than in peripheral blood (Tassone et al. 2004b). All these findings suggest that our current blood mRNA measurements are only an estimate of brain mRNA levels and that CGG repeat number may be more reflective of what is occurring in brain. Additionally, we cannot clarify the possible increases in FMRP levels in lower-repeat premutation carriers and decreases in FMRP levels in higher-repeat premutation carriers without including FMRP data in our analyses. While a quantitative method capable of characterizing the variance in FMRP levels within premutation carriers has been recently developed (Iwahashi et al. 2009), it is still being validated and normed so that FMRP data were not available for the current study. Our results suggest that the current model of the molecular mechanisms at work in the premutation needs to be modified to include the influence of FMRP and explain differences between those with low and high repeat numbers. Further investigations that can include a quantitative measure of FMRP will be instrumental in clarifying the relative roles of increased mRNA and protein changes in the premutation phenotype.

While there is extensive evidence that the premutation is associated with psychopathology (Cornish et al. 2005; Hessler et al. 2005, 2007; Moore et al. 2004a; Roberts et al. 2009), in the current study we found no difference between our premutation and control groups on the global severity index score of the SCL-90-R. Additionally, there was no apparent relationship between SCL-90-R scores and amygdala volume in either the control or the premutation group. One previous study reported a negative correlation between SCL-90-R scores and the activity of the amygdala in response to fearful vs. scrambled faces in those with the premutation (Hessler et al. 2007) but not controls. While our current results cannot speak to potential morphological changes in other brain regions, they do suggest that this relationship was most likely not mediated by volume changes in the amygdala.

It is important to note that in the current study we used both 1.5 T and 3.0 T structural images, which could complicate our interpretation of the structural data. In assessing how combining the images from two scanners at different magnet strength might affect the structural data, we evaluated the scans of six participants who had been scanned in both the 3 T and the 1.5 T scanners to evaluate any potential differences. Two of these six

participants' 3 T scans were also included as controls in our current data set. After co-registering volumes via MAYO BIR Analyze's 3D Voxel co-registration tool, it was noted that there were subtle differences between the two scans when scrutinized side by side. Such issues may be the result of differential spatial warping inherent to each scanner, minor artifacts resulting from re-slicing of different native image resolutions, or even differences in edge contrast. Exploring these factors in-depth was beyond the scope of the current study but may warrant further investigation. To account for any confound that might be introduced into our data set by using images from two machines, a nuisance covariate was used in all analyses including structural data to control for any effect of MRI scanner type on the amygdala or TCV volume measurements.

The current results do not show reduced amygdala volumes in men with the fragile X premutation as a group. Instead, they show a relationship between CGG repeat number and mRNA levels with amygdala volumes only in the men with smaller premutation repeat sizes. These data raise the intriguing possibility that different molecular mechanisms could be affecting brain structure, and potentially function, in those with smaller repeat expansions than those with larger numbers of repeats. Additional research combining structural MRI data with not only CGG repeat and mRNA levels but also careful quantitative measures of FMRP are needed to fully resolve the mechanism that may be driving volumetric brain changes in those with the premutation.

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Fig. 1.

An example of the amygdala profile is shown here traced on one participants brain from a 3 T T1 scan. **a** The amygdala profiles in the coronal view were bounded by the optic tract medially, the substantia innominata dorsally, white matter laterally, and lateral ventricles ventrally in the caudal most portion. As the tracing progresses rostrally, the medial boundary was the semiannular sulcus; dorsolaterally, the boundary was set by the anterior commissure then the ventral putamen followed by the ventral claustrum; laterally, the white matter continuously bounds the amygdala; ventrally, the border was set by the alveus of the hippocampus and ended just lateral of the entorhinal cortex leading toward the lateral

aspects of the uncus. **b** The dorsolateral border of the amygdala was then refined in the transverse view to make sure the putamen was excluded in the most caudal regions. **c** The rostral portion of the amygdala was delineated in the sagittal view by extending the ventral border up to the white matter ascending from the ventral hippocampus towards the dorsal surface of the temporal lobe

Fig. 2. GROUP vs. AMYGDALA VOLUME. Corrected right and left amygdala volumes for both premutation carriers and controls (Box: Mean $\pm$ SE, Whisker: Mean $\pm$ 2*SE). Despite controlling for both age and scanner, no significant differences were found between controls and premutation carriers for both corrected right amygdala volume and corrected left amygdala volume

Fig. 3. CGG REPEAT LENGTH vs. AMYGDALAVOLUME. Partial correlation between CGG repeat number and corrected right amygdala volume in premutation carriers with low repeat numbers (*empty symbols*) and high repeat numbers (*filled symbols*) separated by scanner strength (1.5 T=squares, 3.0 T=triangles). When controlling for age, the low-repeat group (CGG 55 and <85) showed a significant negative correlation between CGG repeat length and corrected right amygdala volume (dashed line, $r=-0.56$). In the high end fragile X premutation group (CGG 85) no significant correlation was found between CGG repeat length and corrected amygdala volume (solid line). Similar patterns were found for when comparing corrected left amygdala volume to CGG repeat length (not shown)

Table 1

Participants' descriptive statistics and FMR1 measures

	Control (<i>n</i> =48)			Premutation (<i>n</i> =49)			<i>P</i> -value
	Mean	SD	Range	Mean	SD	Range	
Age	47.9	16.9	19–76	48.5	16.5	18–78	0.85
WAIS-III full scale	120.5	16.9	84–155	116.2	14.7	81–152	0.19
<i>FMR1</i> CGG repeat size	28.3	4.4	17–42	99.0	37.4	55–199	<0.001
<i>FMR1</i> mRNA	1.4	0.3	0.6–2.0	3.6	1.8	1.7–9.0	<0.001

Control- Missing 1 full scale IQ Premutation-Missing 1 full scale IQ and 4 mRNA

Table 2

Volumetric data

	Controls (<i>n</i> =48)		Premutation (<i>n</i> =49)		<i>P</i> -value
	Mean	SD	Mean	SD	
Total cranial volume (cm ³)	1135397	117958	1123012	135203	0.63
Right amygdala (cm ³)	1903.5	255.2	1883.0	195.2	0.66
Left amygdala (cm ³)	1840.3	250.9	1797.6	186.0	0.34
Corrected right amygdala (cm ³)	0.00168	0.00019	0.00169	0.00019	0.80
Corrected left amygdala (cm ³)	0.00162	0.00019	0.00161	0.00016	0.70

Table 3

Low and high repeat group descriptive statistics and FMR1 measures

	Low (<i>n</i> =22) CGG 55 and <85			High (<i>n</i> =27) CGG 85			<i>P</i> -value
	Mean	SD	Range	Mean	SD	Range	
Age	52.4	18.0	19–78	45.4	14.8	18–73	0.14
WAIS-III full scale	119.0	16.9	81–152	113.8	12.4	83–139	0.23
<i>FMR1</i> CGG repeat size	68.2	8.5	55–81	124.1	32.7	85–199	<0.001
<i>FMR1</i> mRNA	2.4	0.5	1.7–3.8	4.6	1.8	2.8–9.0	<0.001

Low- missing 1 *FMR1* mRNA High- missing 3 *FMR1* mRNA and 1 WAIS-III Full Scale