Decreased Fragile X Mental Retardation Protein Expression Underlies Amygdala Dysfunction in Carriers of the Fragile X Premutation

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Background: The fragile X premutation provides a unique opportunity for the study of genetic and brain mechanisms of behavior and cognition in the context of neurodevelopment and neurodegeneration. Although the neurodegenerative phenotype, fragile X-associated tremor/ataxia syndrome, is well described, evidence of a causal link between the premutation and psychiatric disorder earlier in life, clear delineation of a behavioral/cognitive phenotype, and characterization of the physiological basis of observed symptoms have been elusive.

Methods: We completed functional magnetic resonance imaging targeting the amygdala with an emotion-matching task and concurrent infrared eye tracking, *FMR1* molecular genetic testing, and neuropsychological assessment in 23 men with the premutation (mean age = 32.9 years) and 25 male control subjects (mean age = 30.1 years).

Results: Premutation carriers had significantly smaller left and right amygdala volume and reduced right amygdala activation during the task relative to control subjects. Although both elevated *FMR1* messenger RNA and reduced fragile X mental retardation protein (FMRP) were associated with the reduced activation, multiple regression analysis suggested that reduced FMRP is the primary factor. Premutation carriers also had higher ratings of autism spectrum symptoms than control subjects, which were associated with the reduced amygdala response.

Conclusions: Although prior studies have emphasized a toxic gain-of-function effect of elevated messenger RNA associated with the premutation, the current results point to the role of reduced FMRP in alterations of brain activity and behavior.

Key Words: Amygdala, *FMR1*, fragile X, FXTAS, insula, premutation

Abnormal trinucleotide (CGG) repeat expansions of the fragile X mental retardation 1 (*FMR1*) gene confer significant risk for a surprisingly wide range of health problems, including premature ovarian insufficiency (1,2), neurodegenerative disease (fragile X-associated tremor/ataxia syndrome [FXTAS]) (3), and, of course, fragile X syndrome (FXS), the most frequent known inherited cause of intellectual disability and autism (4). While these three clinical outcomes are well-defined and substantiated by empirical data, other apparent manifestations of *FMR1* mutations, especially those associated with the premutation, appear to have either a variable penetrance (perhaps because of secondary genetic or environmental factors) or a more subtle or subclinical phenotype. Experimenter bias and sampling methods, in some studies, may also lead to discovery of deficits or symptoms mistakenly attributed to the premutation. For example, while studies have suggested higher rates of psychiatric or neurodevelopmental disorders among premutation carriers, these disorders are common in the general population, making the clear attribution of increased risk related to the premutation challenging, especially when studies are small or inadequately controlled. The fragile X premutation occurs in 1 in 113 to 259 female and 1 in 251 to 813 male individuals (5–8); therefore, it is important to understand its potential influence on brain structure and function, as well as on cognition and behavior.

Neuroimaging techniques, such as structural and functional magnetic resonance imaging (MRI), in conjunction with molecular genetic measures specific to the *FMR1* gene, can provide objective methods for investigation of the premutation phenotype and insight into gene-brain-behavior associations explaining a physiological basis of clinical observations. For example, Moore *et al.* (9) reported reduced gray matter volume in the amygdalo-hippocampal complex that was associated with reduced percentage of blood lymphocytes expressing the fragile X mental retardation protein (FMRP) among a group of men with the fragile X premutation. This observation led us to hypothesize that *FMR1*-mediated altered limbic system function may partially explain neuropsychiatric and neurodevelopmental disorders, including autism spectrum disorders (10–13) and anxiety and depression (14–17), that are known to occur in at least a subgroup of premutation carriers.

We conducted a preliminary structural and functional brain MRI (1.5 Tesla) study, focused on the amygdala, in a convenience sample of 12 primarily clinic-referred men with the premutation (mean age 42.9 years) and 13 men without the premutation of similar age, level of education, and intellectual functioning (18). Relative to control subjects, men with the premutation had significantly reduced amygdala activation while passively viewing fearful versus scrambled faces. The reduced amygdala activation was significantly associated with abnormal elevation of *FMR1* messenger RNA (mRNA) in the premutation group, suggesting a dose-dependent pattern in which fold-increase in mRNA was associated with decreased amygdala responsiveness. The clinical relevance of reduced amygdala activation in the premutation carriers was demonstrated by significant correlation with self-reported current psychological symptoms. In light of reports of autism spectrum disorders in young male subjects with the premutation (10–13), we

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suggested that either reduced FMRP or elevated *FMR1* mRNA or a combination of both could contribute to these symptoms via aberrant limbic system function.

The purpose of the present study was to examine amygdala volume and function in a larger sample of nonreferred participants with the premutation relative to control subjects; to determine the relative effects of reduced FMRP and elevated *FMR1* mRNA on these measures of interest; and to study the clinical relevance of amygdala morphology and function, especially with regard to autism spectrum symptomology.

Methods and Materials

Participants

Participants included 23 men with the *FMR1* premutation (mean age 32.9 years) and 25 matched control subjects (mean age 30.1 years). *FMR1* DNA testing was used to confirm allele status for all participants. None of the participants with the premutation were mosaic for either repeat size or methylation—all had between 55 and 200 CGG repeats with no methylation. The control group was matched for age, IQ, level of education, handedness, psychoactive medication use, and ethnicity. Group demographic statistics are shown in Table 1. Male subjects with the premutation were selected from fragile X pedigrees either because 1) they were known to carry the premutation (and confirmed by *FMR1* DNA testing) or 2) they were untested sons of female individuals with the premutation who were later confirmed through study testing. None was ascertained or referred to the clinic due to clinical symptoms. Neurologic examinations, including assessment of possible FXTAS, were normal for all participants. Exclusion criteria included any acute medical condition such as renal, liver, or cardiac disease; migraine headache; history of head trauma; history of alcoholism or drug abuse; and use of current medications that affect cerebral blood flow. Two participants with the premutation were included in a previous study (16).

Neuropsychological Assessment

Cognitive ability was based on Full Scale IQ using the Wechsler Adult Intelligence Scale, Third Edition (19). Autism phenotype features were assessed with the Autism Diagnostic Observation Schedule, Module 4 (ADOS) (20) and the Social Responsiveness Scale (SRS), adult version (21).

Table 1. Molecular Genetic, Demographic, and Clinical Descriptive Statistics by Group

	Control Subjects ($n = 25$)	Premutation ($n = 23$)	Significance
CGG Repeats	28.32 (3.56)	106.91 (40.0)	$p < .001$
Range	19–33	55–199	
FMR1 mRNA	1.46 (.28)	3.65 (1.71)	$p < .001$
Range	1.01–1.93	2.16–8.33	
FMRP[a]	112.81 (95.36)	66.29 (51.95)	$p = .034$
Range	13.40–473.60	14.50–182.60	
Handedness (RH)	80.0%	95.5%	ns
Psychoactive Medication	33.3%	28.6%	ns
Age	30.12 (7.75)	32.95 (8.89)	ns
Caucasian	68.5%	82.6%	ns
Years of Education	15.39 (3.60)	15.47 (2.97)	ns
Full Scale IQ	125.52 (17.77)	118.00 (16.05)	ns
SRS Total	27.96 (23.75)	39.61 (31.95)	$p = .16$
ADOS Total	2.36 (2.74)	5.87 (6.42)	$p = .01$

ADOS, Autism Diagnostic Observation Schedule; FMRP, fragile X mental retardation protein; IQ, intelligence quotient; mRNA, messenger RNA; ns, nonsignificant; RH, right handed; SRS, Social Responsiveness Scale.

[a]FMRP raw data are shown. Log transformation was used to normalize distributions for *t* test of group differences.

Molecular Genetic Measures

CGG Repeat Size. Genomic DNA was isolated from peripheral blood lymphocytes using standard methods (Qiagen, Valencia, California). Repeat size and methylation status were determined using both polymerase chain reaction and Southern blot analysis using an Alpha Innotech FluorChem 8800 Image Detection System (San Leandro, California) as previously described (22,23).

***FMR1* mRNA.** All quantifications of *FMR1* mRNA were performed using a 7900 Sequence detector (PE Biosystems, Foster City, California) as previously described (24).

FMRP Levels. Fragile X mental retardation protein was quantified in lymphocytes utilizing a recently described sandwich enzyme linked immunosorbent assay (ELISA) for FMRP, as described previously (25). The FMRP ELISA assay differs from the commonly used immunocytochemistry method in that the ELISA approach provides a quantitative measure of FMRP level, whereas the immunocytochemistry method does not measure protein level, only the proportion of cells with detectable staining.

MRI Acquisition

Images were acquired on a 3.0T Siemens scanner with Echospeed gradients and a Siemens (Malvern, Pennsylvania) 8-channel whole head coil. Functional magnetic resonance imaging (fMRI) was performed using a single-shot gradient-recalled echo-planar imaging sequence that is automatically corrected for motion and magnetic field distortions, utilizing a point spread function mapping approach (26). Thirty-eight slices (3.4 mm thick), aligned 30° clockwise from parallel to the anterior and posterior commissure plane and covering the whole brain, were imaged with a temporal resolution of 2 seconds using a T2* weighted gradient echo-planar pulse sequence with echo time 13 milliseconds, flip angle 84°, field of view 220 mm, and base resolution 64. For the purpose of manual segmentation and to aid in localization, co-registration, and normalization of functional data, a high-resolution T1-weighted magnetization prepared rapid acquisition gradient-echo sequence of 192 slices with repetition time 2170 milliseconds, echo time 4.82 milliseconds, field of view 256 mm, and 1.0 mm slice thickness was acquired.

Image Preprocessing

Preprocessing of the imaging data was completed using statistical parametric mapping software (SPM5; Wellcome Department of Imaging Neuroscience, University College London, United Kingdom). Images were corrected for movement using least squares minimization without higher-order corrections for spin history and normalized to stereotaxic Montreal Neurological Institute, then resampled every 2 mm using fourth degree B-spline interpolation and smoothed with a 5 mm Gaussian kernel to decrease spatial noise.

Amygdala Volume

Amygdala volumes were quantified by operator-guided manual segmentations using Mayo Biomedical Imaging Resource Analyze 8.5 and 9.0 (AnalyzeDirect, Inc., Overland Park, Kansas) (27–29). These guidelines were developed from the anatomical analysis of postmortem human brains using histological sections of tissue cut perpendicular to the hippocampus axis. For a detailed description of this protocol, see Schumann *et al.* (30). Amygdala volumes were corrected for total cerebral volume. The reliability between tracers on 10 cases was 94.7% for the left amygdala and 96.9% for the right amygdala.

Total Cerebral Volume

To obtain a measure of total cerebral volume, each series of images was edited manually using Mayo Biomedical Imaging Resource Analyze 8.5 and 9.0 (27–29) to remove nonbrain structures, the brainstem, and the cerebellum. Using a Gaussian cluster multispectral thresholding tool, the ventricles were defined and excluded.

fMRI Emotion Processing Task

The emotion processing task was modeled after a task used by Brown *et al.* (31). Participants viewed three faces expressing either fear or anger, where the emotion presented at the bottom of the screen was identical to one of the two emotions presented above. Participants were asked to press a button to indicate which top emotion matched the sample, while accuracy and reaction time were recorded. Faces were taken from two standard sets of pictures of facial affect (32,33) and presented in grayscale with hair and other nonface features cropped. Fearful and angry expressions are perceived as threatening and robustly engage the amygdala (34,35). Control stimuli consisted of three simple geometric shapes (squares, circles, and ovals), colored in grayscale gradients matching the face stimuli in average luminance and arranged in the same configuration. Subjects matched the stimuli similarly to the emotion trials. Each set of three images, whether emotions or objects, was presented for 3 seconds in a pseudorandom order (Figure 1). Intervals between stimuli were jittered between 1 and 5 seconds. A total of 104 face trials and 104 control trials were presented over two runs.

Eye Tracking

Eye-tracking data were collected concurrent with the fMRI emotion-matching task using a long-range infrared eye tracking system (Applied Science Laboratories, Bedford, Massachusetts) and processed using Eyenal 6.0 (Eyetracker, North Sydney, Australia). Areas of interests were rectangles covering all three face or shape ovals individually.

fMRI Analysis

Statistical analysis was performed on individual and group data using the general linear model and the theory of Gaussian random fields as implemented in SPM5 (36) with one predictor (convolved with a standard canonical hemodynamic response function) for each condition. For both within-group and between-group comparisons, significant clusters were defined as those that exceeded a threshold value equivalent to a one-tailed t test $p < .01$ for a higher level of quality control, corrected false discovery rate (FDR) on the voxel level (37). Once thresholded, activation foci were superposed on normalized and averaged high-resolution magnetization prepared rapid acquisition gradient-echo images; their locations were identified both manually using an atlas with known neuroanatomical landmarks (38) and automatically using xjView (http://www.alivelearn.net/xjview8/).

A within-subjects procedure was used to model all the effects of interest for each subject by contrasting experimental and control trials. This model estimates the error variance for each condition of interest across participants rather than across scans (39). These contrast images generated previously on the individual level were analyzed using a general linear model to determine voxel-wise t statistics and generating one contrast image per participant, per effect of interest.

Within-group analyses of each contrast were performed to indentify brain regions showing similar response modulations across participants in both the premutation and control groups for each contrast. Between-group analyses were then performed to determine differences in average activation responses to each contrast between the two groups.

Region of interest (ROI) analyses for the amygdala and insula were carried out using MarsBar toolbox for SPM (40). Contrasts defined remained the same as previously described. Each contrast of interest was analyzed only in voxels that fell either within the Montreal Neurological Institute template (41) of the area of interest (e.g., insula) on normalized images or within boundaries defined by operator-guided manual segmentations of the individual specific amygdala, as described above, applied to individual unnormalized functional images. A t statistic termed contrast value was then calculated as the average of all voxels' contrast values that fell within the defined ROI. The contrast value in these analyses is comparable with the Z score reported in the whole-brain analyses tables. The contrast values were then used in between-group independent sample T-tests and Spearman correlation and regression analyses in conjunction with biological measures

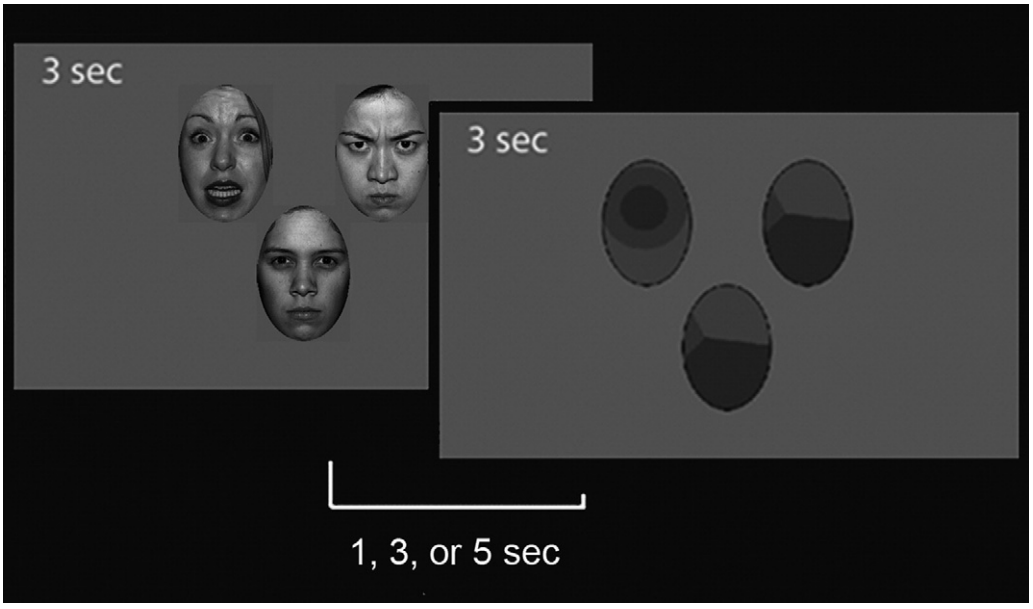

Figure 1. Sample trials of the emotion-matching task (33). Participants matched the bottom emotion to either the top left or top right emotion. For the control trials, participants matched the shapes.

and neuropsychological data. In all analyses utilizing ROI-derived data, outlier values of greater than 1.5 interquartiles from the median of the sample were excluded from analysis.

Results

Participant *FMR1* genetic, demographic, and clinical descriptive statistics are shown in Table 1. The FMRP distribution was positively skewed and therefore log-transformed to achieve normality. Fragile X mental retardation protein was reduced by 12% in the premutation group relative to control subjects, $t(38) = 2.19$, $p < .034$. Premutation carriers had significantly higher ADOS total scores than control subjects, with mean level (5.87) somewhat below the autism spectrum cutoff (7). Two control subjects and six premutation carriers had ADOS scores beyond the autism spectrum cutoff. On the SRS, reported by a spouse or significant other, premutation carriers had elevated scores, but this difference did not reach significance.

Total Cerebral and Amygdala Volume

Independent samples *t* tests revealed that premutation carriers had significantly smaller total amygdala volume than control subjects for both raw [$t(46) = 2.328$, $p = .024$] and corrected [$t(46) = 2.129$, $p = .039$] volumes (Figure 2; Supplement 1). This amygdala volume reduction in the corrected data was stronger on the right [$t(46) = 2.073$, $p = .044$] but was still marginally significant on the left [$t(46) = 1.984$, $p = .053$]. Both raw values for right [$t(46) = 2.033$, $p = .048$] and left [$t(46) = 2.267$, $p = .028$] amygdala were significantly smaller in the premutation group. Total cerebral volume did not differ between the two groups [$t(46) = -.423$, $p = .674$].

In the premutation carrier and control groups, all correlations between *FMR1* molecular measures and corrected amygdala volumes were nonsignificant.

Emotion-Matching Task Performance

There were no between-group differences for percentage correct in response accuracy [premutation mean = 81.4%, control mean = 78.8%, $t(44) = -.952$, $p = .346$] or reaction time [premutation mean = 2.18 sec, control mean = 2.20 sec, $t(45) = .210$, $p = .835$] during the emotion-matching task.

Eye-Tracking Results

There were no group differences in the average looking time per trial for the defined areas of interests covering the faces or proportion of looking time at faces (premutation mean 1.40 seconds and 75.75%, control mean 1.40 seconds and 71.74%, respectively) or shapes (premutation mean 1.14 seconds and 70.09%, control mean 1.08 seconds and 63.72%, respectively), all $p > .17$.

Amygdala and Associated Brain Activation

Within-Group Analysis. When matching emotional faces compared with matching shapes (emotion faces > shapes), the premutation group showed similar brain activation patterns compared with control subjects at a thresholding of $p < .01$ with FDR correction (Supplement 1). Both groups showed robust activation bilaterally in the insula, mid cingulate gyrus, fusiform gyrus, parietal lobe, superior frontal gyrus, and middle frontal gyrus. Both control subjects and premutation carriers activated the amygdala bilaterally, which was evident in the normalized whole brain analysis (Figure 3).

Between-Group Analysis. A direct comparison between premutation and control groups for the emotion faces > shapes contrast across the whole brain showed no significant activation differences in either direction (premutation > control or control > premutation) using an FDR correction at $p < .01$. Region of interest analyses, where activity specific to each participant was extracted from manually traced amygdala ROIs, revealed that left amygdala activation was significantly less for the premutation group [$t(44) = 2.161$, $p = .036$] (Supplement 1). The group difference in the right amygdala was nonsignificant [$t(44) = 1.43$, $p = .160$]. No significant group differences were observed in the insula (all $p > .80$).

Molecular and Functional MRI Correlation Analysis

As expected in the premutation group, CGG repeat number was strongly and positively correlated with *FMR1* mRNA expression ($r = .842$, $p < .001$). There was also a negative trend for FMRP with increasing *FMR1* mRNA concentration (rho = −.482, $p = .069$), as expected for

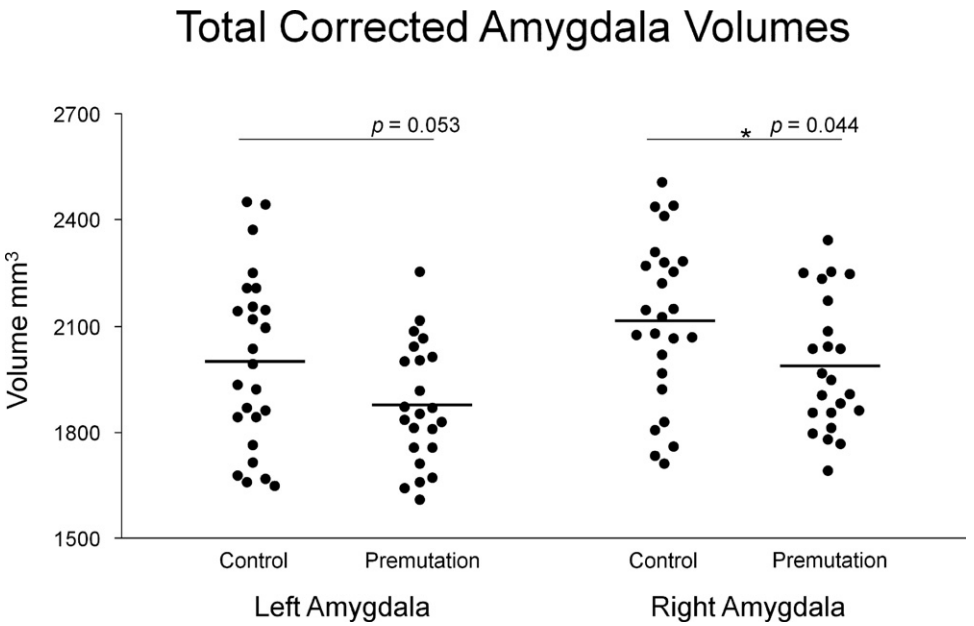

Figure 2. Distributions of left and right amygdala volume (corrected for total cerebral volume) in premutation carriers and control subjects.

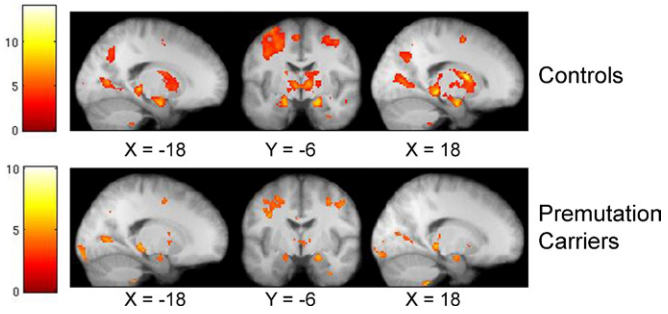

Figure 3. Whole-brain within-group analysis in the emotion > control condition for control subjects and premutation carriers. Significant clusters were thresholded on the voxel level at $p < .01$ after false discovery rate correction. Both groups significantly activated the bilateral amygdala during this task. Bars at left represent degree of activation using the *t* statistic.

reduced efficiency of translational initiation with increasing repeat size in RNA (42).

FMR1 mRNA was correlated negatively with right amygdala activation (ROI analysis; rho = −.625, $p = 0.002$) and with right insula activity (rho = −.610, $p = .003$) in the emotion faces > control contrast. Fragile X mental retardation protein correlated positively with both right (rho = .697, $p = .003$) and left (rho = .771, $p < .0001$) amygdala activation (Figure 4) and with right insula activation (rho = .524, $p = .037$). No significant correlations between activation of these brain regions and the genetic measures were observed in the control group.

Because we observed strong associations between reduced amygdala activation and both elevated *FMR1* mRNA and decreased FMRP in premutation carriers, we sought to determine the relative and independent effects of these factors in multiple regression analyses ($n = 16$). Using right and left amygdala activation as the dependent variables and FMRP and *FMR1* mRNA as independent variables, we found that FMRP was positively correlated with activation (left, $\beta = .681$, $p = .009$; right, $\beta = .572$, $p = .023$), whereas *FMR1* mRNA showed no significant association (left, $\beta = .024$, $p = .915$; right, $\beta = -.327$, $p = .158$). Analogous regression analyses with left and right insula activation demonstrated no significant associations with *FMR1* mRNA and an association between FMRP and right insula activation that approached significance ($\beta = -.490$, $p = .055$).

Neuropsychological Correlates of Amygdala Activation

The planned correlations between amygdala activation for the emotion > shapes contrast and SRS total score were significant in the premutation group only for the right amygdala ROI (rho = −.469, $p = .032$). Post hoc exploratory correlations using the subscales of the SRS showed that reduced right amygdala activation was most strongly associated with problems in social information processing (rho = −.588, $p = .005$) and reciprocal social communication (rho = −.497, $p = .022$), although similar correlations were observed for the left amygdala. No other significant correlations between amygdala activation and clinical measures were observed, including those with IQ and ADOS scores.

Discussion

The results of this study demonstrate that relative to control subjects, men with an *FMR1* premutation allele had smaller amygdala volumes and reduced amygdala activation during an emotion-matching task. In contrast to our prior study (18), in this investigation, we were able to determine that aberrant amygdala function was more strongly associated with reduced FMRP (the cause of FXS) than abnormal elevation of *FMR1* mRNA (the hypothesized toxic mRNA gain-of-function model underlying FXTAS). To our knowledge, this the first evidence of the impact of reduced FMRP without methylation in *FMR1* premutation carriers. Standardized clinical assessment indicated that men with the premutation had more significant communication and reciprocal social behavior symptoms of autism spectrum disorder compared with control subjects; however, on a group level, the severity of these symptoms did not reach clinical significance. Exploratory analyses showed that the FMRP-mediated blunted amygdala response may be correlated with deficits in social information processing, a component of the broader autism phenotype. Because no evidence of transcriptional silencing was observed in any individual with a premutation included in this study, who do show levels of *FMR1* mRNA substantially higher than normal levels (24,43,44), the mild reductions in FMRP with increasing CGG repeat length (45–48) are due primarily to reduced efficiency of translational initiation (42). Although the full range of FMRP levels in individuals with the full mutation, premutation, and general population control subjects has not been fully elucidated, it is important to emphasize that the relatively lower FMRP levels observed in some premutation carriers in this study are likely to be quite modest compared with most male subjects with the full mutation and FXS who express essentially no or very little FMRP and who demonstrate more substantial behavioral and cognitive deficits.

Overall, the study indicates that neurodevelopmental and psychiatric symptoms previously reported among younger men and boys with the premutation may be due to the same molecular genetic mechanism leading to FXS but to a milder degree. Previous findings, including our own (16,18), highlighting the potential negative impact of elevated mRNA on these symptoms suggests that both mRNA- and protein-level effects may be operating in the premutation range, with protein decrements perhaps playing a larger role, at least in amygdala-mediated functions.

In the current cohort of participants, we found reduced amygdala volumes in premutation carriers, whereas in our prior

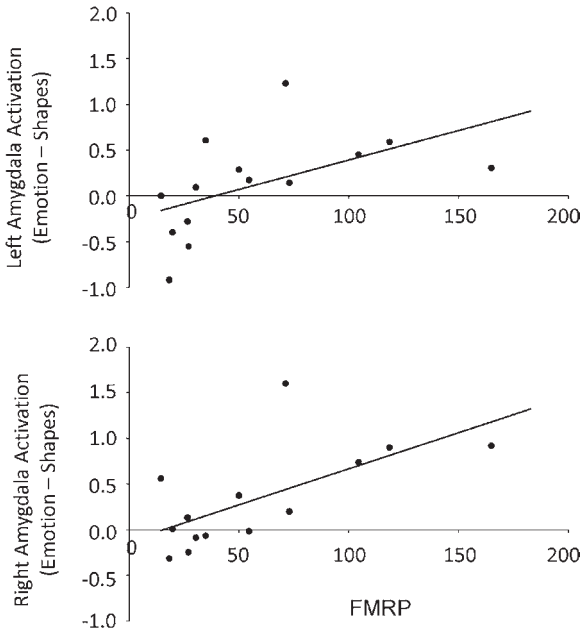

Figure 4. Scatter plots showing the association between right (top) and left (bottom) amygdala activation (contrast value extracted from region-of-interest analyses) and fragile X mental retardation protein in premutation carriers. FMRP, fragile X mental retardation protein.

study of a different group of participants (18), we did not find this effect. A number of factors might explain this difference. For example, the more powerful 3T scanner used here provided higher signal-to-noise ratio, enabling higher levels of image quality, speed, and resolution, perhaps making the group effect easier to detect. Also, somewhat larger sample sizes in the present study provided additional data and greater power to detect differences. However, consistent with the current results is the fact that reduced amygdala volume is now a replicated finding in individuals with the fragile X full mutation— both toddlers (49) and older children and adolescents (50)—and these individuals have substantial FMRP deficits.

Prior fMRI studies in FXS using social stimuli (human faces showing direct or averted gaze) (51) have documented greater sensitization in the left amygdala with successive exposure to direct gaze in a sample of adolescent boys. Given that patients with FXS and some individuals with the premutation have reduced FMRP (albeit to a different degree), we might expect premutation carriers to have a similarly increased amygdala response. The explanation for this inconsistency is unclear but could be related to the nature of the tasks, the added complexity of mildly reduced FMRP and elevated message in premutation carriers, or due to developmental differences. Nevertheless, this research highlights the critical role of amygdala function in perception of and response to human social stimuli and in social behavior in individuals affected by *FMR1* mutations.

In our previous report, we suggested that the altered limbic system function observed in men with the premutation could reflect early prodromal brain changes associated with later-onset FXTAS. However, given that the primary molecular genetic mechanism underlying FXTAS neuropathology is thought to involve abnormal elevation of the expanded CGG-repeat *FMR1* mRNA, our current results suggest that lowered FMRP contributes to amygdala dysfunction and that the latter effect could be both neurodevelopmental in origin and independent of FXTAS progression.

The reduced amygdala activation in premutation carriers observed here cannot be explained by decreased visual fixation on stimuli, as we observed similar fixation patterns between groups. Recruitment biases are unlikely to have affected our findings because participants were selected at random from pedigrees and were not clinic referred. The blunted amygdala response cannot be explained by reduced amygdala volume, as the ROI method controls for individual differences in amygdala size and, additionally, we did not find a significant correlation between volume and activation (data not shown). Interestingly, although differences in brain activation were observed during the emotion-matching task, we found no difference in performance, indicating that brain activation patterns may be more sensitive to effects of the premutation than observable behavior.

There were important limitations to the study. Although we took several steps to minimize recruitment bias, this form of bias cannot be completely eliminated because individuals make decisions whether to participate based on many factors, some of which may be associated with effects of the premutation being studied here. Although men with the premutation were rated as having higher rates of autism symptoms on clinical exam, it is not possible to rule out effects of experimenter bias. Many participants were assessed blind to group; however, blinding was not possible in all cases because of personal information revealed during the interview portions of the ADOS. Approximately one third of participants in each group were taking psychoactive medication, which may have influenced our results and somewhat detracts from the generalizability of the findings. It is important to emphasize that the *FMR1* molecular genetic measures were ascertained from blood samples and may not necessarily reflect what would be found in brain tissue. Finally, we were unable to derive valid FMRP values for all participants in the study; as such, the results pertaining to the association between FMRP level and brain function must be considered preliminary.

Our findings may have treatment implications for some individuals with the premutation who present with significant symptoms of FXS or ASD. The metabotropic glutamate receptor theory of FXS states that mental impairment and phenotypic behaviors associated with FXS arise, at least in part, from constitutive activation of translational pathways normally controlled by group 1 metabotropic glutamate receptor (metabotropic glutamate receptor 1 and metabotropic glutamate receptor 5 [mGluR5]) activity (52). Accordingly, downregulation of mGluR5 activity, both pharmacologically and genetically, has rescued many phenotypes examined in the animal models of FXS (53). Phase II clinical trials of mGluR5 antagonists in patients with FXS are now ongoing, and it is intriguing to consider whether premutation carriers with significantly lowered FMRP would benefit from these treatments as well.

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