The effect of pre-mutation of X chromosome CGG trinucleotide repeats on brain anatomy

Caroline J. Moore,1 Eileen M. Daly,1 Flora Tassone,7 Carolyn Tysoe,4 Nicole Schmitz,1 Virginia Ng,3 Xavier Chitnis,2 Philip McGuire,2 John Suckling,5 Kay E. Davies,6 Randi J. Hagerman,8 Paul J. Hagerman,7 Kieran C. Murphy1 and Declan G. M. Murphy1

1Division of Psychological Medicine and 2Neuroimaging Research Group, Department of Neurology, Institute of Psychiatry, King’s College London, DeCrespigny Park, 3Neuroimaging Department, Maudsley Hospital, Denmark Hill, London, 4Medical Genetics Service for Wales, University Hospital of Wales, Heath Park, Cardiff, 5Brain Mapping Unit, Department of Psychiatry, University of Cambridge, Addenbrooke’s Hospital, Cambridge 6Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, UK, 7Department of Biological Chemistry, University of California, Davis and 8The MIND Institute and Department of Pediatrics, UC Davis Medical Center, Sacramento, CA, USA

Correspondence to Professor Declan Murphy, Section of Brain Maturation, Division of Psychological Medicine, Institute of Psychiatry, King’s College London, DeCrespigny Park, London, UK
E-mail: sphadgm@iop.kcl.ac.uk

Summary
Expanded trinucleotide repeats are associated with several neuropsychiatric disorders, including fragile X syndrome (FraX) which is the most common inherited form of mental retardation. It is currently thought that FraX results from having >200 CGG trinucleotide repeats, with consequent methylation of the fragile X mental retardation gene (FMR1) and loss of FMR1 protein (FMRP). Pre-mutation carriers of FraX (with 55–200 CGG trinucleotide repeats) were originally considered unaffected, although recent studies challenge this view. However, there are few studies on the effect of pre-mutation trinucleotide repeat expansion on the male human brain using quantitative MRI. Also the results of prior investigations may be confounded because people were selected on the basis of clinical and neurological features, and not genetic phenotype. We compared the brain anatomy of 20 adult male pre-mutation members of known FraX families with 20 healthy male controls. The two groups did not differ significantly in age, intelligence quotient (IQ) or handedness. We also investigated whether any observed effects were associated with: (i) ageing; (ii) expansion of pre-mutation CGG trinucleotide repeats; (iii) reduction in the percentage of lymphocytes staining with anti-FMRP antibodies [%FMRP(+) lymphocytes]; and (iv) elevation of FMR1 mRNA levels. Male pre-mutation carriers of FraX, compared with matched controls, had significantly less voxel density in several brain regions, including the cerebellum, amygdalo-hippocampal complex and thalamus. Within pre-mutation carriers of FraX, ageing, increases in the number of CGG trinucleotide repeats and decreases in %FMRP(+) lymphocytes were associated with decreasing voxel density of regions previously identified as decreased relative to controls. Regional grey and white matter density is significantly affected in male pre-mutation carriers of FraX recruited on the basis of genetic, not clinical, phenotype. The association of voxel density reduction and ageing is consistent with observations of a subgroup of older pre-mutation males who present with cognitive decline. Moreover, our findings suggest, for the first time, an association between voxel density reduction and genetic variation in FraX.

Keywords: pre-mutation; trinucleotide repeats; fragile X syndrome; X chromosome; brain, MRI

Abbreviations: FMR1 = fragile X mental retardation gene; FMRP = FMR1 protein; FraX = fragile X syndrome; %FMRP(+) lymphocytes = percentage of lymphocytes staining with anti-FMRP antibodies; IQ = intelligence quotient.


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Introduction

Expanded trinucleotide repeats are associated with disabling neuropsychiatric and neurological disorders, such as Huntington’s disease, myotonic dystrophy, spinal and bulbar muscular atrophy and fragile X syndrome (FraX). The clinical phenotype of FraX results from having >200 CGG trinucleotide repeats, with subsequent methylation of the fragile X mental retardation gene (FMR1) and loss of FMR1 protein (FMRP) production (Verkerk et al., 1991; Yu et al., 1991).

Pre-mutation carriers of FraX have 55–200 CGG trinucleotide repeats. Current thinking suggests that the distinction between full and pre-mutation FraX is less clear than once thought (Hagerman and Hagerman, 2002), challenging an assumption that pre-mutation carriers of FraX are unaffected. For example, premature ovarian failure (Allington-Hawkins et al., 1999) has been observed in some female pre-mutation carriers of FraX. Molecular genetic investigations of male pre-mutation carriers of FraX have reported diminished production of FMRP in blood (Tassone et al., 2000a,b) and lymphoblastoid lines harbouring pre-mutation alleles (Kenneson et al., 2001); and elevated levels of FMR1 mRNA (Tassone et al., 2000b,c; Hagerman et al., 2001; Kenneson et al., 2001). There is also evidence that differences in CGG trinucleotide repeat sizes within the pre-mutation range may affect clinical involvement. For example, reduced FMRP production is consistent only at the higher end (>100 CGG trinucleotide repeats) of the pre-mutation range (Tassone et al., 2000a,b; Kenneson et al., 2001); and female carriers with >100 CGG trinucleotide repeats may have more ‘emotional problems’ than those with <100 CGG trinucleotide repeats (Johnston et al., 2002).

A neurological condition involving tremor and/or ataxia and cognitive decline has also been reported in a subgroup of older pre-mutation male carriers of FraX (Hagerman et al., 2001), and it has been suggested that this is a consequence of either a mild deficit of FMRP or an elevation of FMR1 mRNA. A recent MRI investigation of 17 male pre-mutation carriers of FraX who were studied because they displayed ataxia, tremor, rigidity and cognitive dysfunction reported that pre-mutation carriers (compared with age-matched controls) had significantly greater atrophy of the cerebral cortex and cerebellum, ventricular enlargement, and thinned corpus callosums (Brunberg et al., 2002). Whilst this study was an important first step, it may have been confounded as people were selected because they demonstrated clinically detectable abnormalities. Nonetheless, it is of interest that patients presented initial signs of intention tremor in their 50s or 60s, raising the question of whether this condition emerges as pre-mutation FraX carriers get older.

There has been only one investigation, using in vivo brain imaging, of pre-mutation carriers of FraX recruited on the basis of genetic, not clinical, phenotype. We reported (Murphy et al., 1999) that female carriers have significant differences compared with controls in: (i) volume of hippocampus, caudate nucleus, thalamus, peripheral CSF and third ventricle; (ii) glucose metabolism of hippocampi, cerebellum and temporal, parietal and occipital association cortices; and (iii) right–left metabolic asymmetry of Wernicke’s and Broca’s language areas. Our prior findings, however, may have been confounded by Lyonization (i.e. the presence of an inactivated second X chromosome in females) and subsequent activation ratios (i.e. the proportion of active X chromosomes that have an affected allele).

Here we studied male pre-mutation carriers to eliminate the effect of Lyonization that might otherwise have obscured correlations between brain anatomy and other variables (such as CGG trinucleotide repeat length). We compared the brain anatomy of male pre-mutation carriers of FraX with controls using quantitative structural MRI. We recruited men based only on their genotype, and not on physical or cognitive phenotype; the first time such selection has been used in neuroanatomical investigations of pre-mutation FraX. Our design allowed us additionally to investigate the association between any observed effects and: (i) intelligence quotient (IQ); (ii) ageing; (iii) expansion of CGG trinucleotide repeat number; (iv) reduction in the percentage of lymphocytes staining with anti-FMRP antibodies [%FMRP(+) lymphocytes]; and (v) elevation of FMR1 mRNA levels.

Subjects and methods

Subjects

Male pre-mutation FraX carriers were recruited from genetic services throughout Britain, on the basis of genetic, not clinical, phenotype, and were members of known FraX families. All subjects gave informed consent to participate in this study, which was approved by the ethical committee of the Institute of Psychiatry and the South London and Maudsley NHS Trust, and the individual local research ethics committees attached to the genetic centres where subjects were recruited.

All participants in the study underwent routine blood tests, structured physical and psychiatric examination (Murphy et al., 1996, 1997) (for the presence of DSM-IV axis 1 or 2 disorder: American Psychiatric Association, 1994), and clinical MRI to exclude gross structural pathology. Full-scale IQ was measured using the Wechsler Adult Intelligence Scale (Wechsler, 1987), and handedness was determined using Annett’s questionnaire (Annett, 1970).

Blood testing

Polymerase chain reaction (PCR) analysis (Brown et al., 1993) confirmed pre-mutation FraX (55–200 CGG trinucleotide repeats) and control (<50 CGG trinucleotide repeats) status. Further investigation of carriers gave the CGG trinucleotide repeat number (n = 19), %FMRP(+) lymphocytes (n = 18) and mRNA elevation (n = 17), with n subject variation due to sample decay. A ‘Fragile X Size Polymorphism Assay’ kit (Applied Biosystems) measured CGG trinucleotide repeat number. Previously published methods were used to measure
%FMRP(+) lymphocytes (Willemsen et al., 1995, 1997; Tassone et al., 2000a) and mRNA levels (Tassone et al., 2000b) in pre-mutation carriers.

In brief, %FMRP(+) lymphocytes were determined by immunocytochemistry of blood smears (Willemsen et al., 1995, 1997; Tassone et al., 2000a), using the indirect alkaline phosphatase staining technique for detection. Cells were fixed with 3% paraformaldehyde and permeabilized with methanol. Antibody detection involved incubation with FMRP-specific antibody IC3a, followed by incubation with goat anti-mouse immunoglobulin conjugated with biotin (DAKO) and a final incubation with streptavidin-biotinylated alkaline phosphatase (DAKO). A fuschsin substrate chromagen system (DAKO) allowed visualization of antibody-bound FMRP. For each sample collected, 200 lymphocytes were counted and %FMRP(+) lymphocytes were scored for the presence of a red-staining cytoplasmic ring that indicates the presence of FMRP. It is important to note that this method is not a quantitative measure of FMRP in each cell; rather, it provides the percentage of the cells expressing FMRP. Although we only investigated %FMRP(+) lymphocytes for the pre-mutation carriers of FraX, this test does have some variability in control populations. For example, a study of 33 males with non-expanded alleles demonstrated a mean %FMRP(+) lymphocytes of 89 ± 9% (Willemsen et al., 1997).

FMR1 mRNA was quantified using an automated, fluorescence detection reverse transcription (RT)–PCR assay. An 81 bp amplicon derived from β-glucuronidase (GUS) mRNA (GenBank accession number NM000181) was used as a reference. GUS mRNA is a convenient reference, since normal peripheral blood leukocytes maintain comparable levels of FMR1 and GUS mRNAs. For each blood sample, quantitative fluorescence RT–PCRs were performed in duplicate for each starting total RNA concentration, for both FMR1 and GUS amplicons. Secondary control reactions for both FMR1 and GUS were run in parallel using a standard lymphoblastoid line (RMM 7666; ATCC CCL114). Fluorescence changes were monitored for each reaction in real time using a 7700 Sequence Detector (PE Biosystems). The extent of fluorescence is directly proportional to DNA copy number, and relative mRNA levels were determined by comparison of the cycle numbers for which the relative fluorescence signal exceeded a designated threshold value.

We investigated whether our findings supported previous studies, which showed diminished levels of FMRP in pre-mutation carriers of FraX in blood (Tassone et al., 2000a,b) and elevated levels of FMR1 mRNA (Tassone et al., 2000b,c; Hagerman et al., 2001; Kenneson et al., 2001) with increasing CGG trinucleotide repeat expansion. Such correlations were investigated using Kendall’s tau (since not all variables were normally distributed).

**Scanning protocol**

MRI scans of the whole head were acquired on a 1.5 T neuro-optimized MR system (GE Medical Systems, Milwaukee, WI). The structural MRI sequence employed was a 3D-Spoiled Grass (SPGR) volume scan. There were 124 contiguous slices (256 × 192), a 1.5 mm slice thickness (with no gap), a field of view of 220 mm, a TR (repetition time) of 13.8 ms and a TE (echo time) of 2.8 ms.

**Automated computational analysis of MRI scans**

Pre-statistical image processing methods (Ashburner and Friston, 2000) were employed using Statistical Parametric Mapping (SPM99) software. In brief, scans were normalized to a template brain approximating the standard space of Talairach and Tournoux (1988) via a 12 parameter affine registration and a set of low frequency cosine basis functions (Ashburner and Friston, 2000). Images were segmented into probabilistic maps of grey matter, white matter and CSF by a modified mixture-model clustering algorithm (Ashburner and Friston, 2000). The segmented images were then smoothed using a 2D Gaussian filter with SD = 2 mm (Bullmore et al., 1999). This smoothing takes a local average of voxel values, under the smoothing kernel. This converts the grey matter segments into images representing the local proportion of brain tissue that has been classified as grey matter. The smoothing also serves to reduce the effects of individual variation in sulcal/gyral anatomy.

**Statistical analysis**

The data were analysed with our own in-house statistical package, which has been validated (Bullmore et al., 1999), and used previously in the study of brain structure (Sigmundsson et al., 2001).

Regional between-group differences in grey and white matter and the relationships between brain structure and genetic variables were investigated by regression of an appropriate general linear model at each intracerebral voxel. Inference was via a permutation distribution of spatially informed statistics (Bullmore et al., 1999) with significance levels set to control for multiple comparisons by having less than one estimated false-positive region (clusters) across the image.

In brief, the processing proceeded as follows. Maps of the standardized general linear model coefficient of interest (group membership or genetic variable) $\beta$ at each voxel were thresholded such that only voxels with $P(\beta) < 0.05$ were retained. The sum of voxelwise statistics for each three-dimensional suprathreshold cluster was the test statistic, the sign indicating a relative excess or deficit in local tissue density. Significance testing of the clusters was performed using a null distribution of this test statistic similarly obtained after repeatedly randomly permuting the relevant factor in the general linear model and refitting of the model (Bullmore et al., 1999).

**Between-group differences**

We investigated group differences in grey and white matter voxel density for all pre-mutation carriers of FraX relative to...
controls using our in-house statistical package (Bullmore et al., 1999). Main effects identified regions where grey and white matter voxel densities for carriers were (i) greater than or (ii) less than the corresponding voxel densities for controls. Results are reported at a significance level of $P < 0.001$ (i.e. $< 1$ false-positive cluster expected).

Between-group differences in (i) full-scale IQ; (ii) verbal IQ; (iii) performance IQ; and (iv) ageing were investigated by correlating the voxel density values (for each cluster identified in the group analysis described above) with the variable of interest in SPSS Version 11.0 for Windows. Pearson’s $r$ correlation statistics were then converted into Fisher’s $z$-score ($z_{obs}$), which assessed whether correlations differed significantly (Pallant, 2001). For differences to be significant, $z_{obs}$ must be $\leq -1.96$ or $\geq 1.96$; only these are reported.

**Genetic variables within subject group for carriers of FraX**

Having identified significant group differences, correlations were performed using our in-house statistical package (Bullmore et al., 1999), within pre-mutation carriers of FraX to test the association between decreased voxel density and: (i) CGG trinucleotide repeat expansion; (ii) %FMRP(+) lymphocytes; and (iii) $FMR1$ mRNA elevation. Correlations were only performed within pre-mutation carriers of FraX, since our interest was to investigate regions shown to differ between pre-mutation carriers of FraX and controls.

It was predicted that, within regions identified in the main effects, there would be correlations between previously observed effects on voxel density and: (i) expansion of CGG trinucleotide repeat size; (ii) %FMRP(+) lymphocytes; and (iii) $FMR1$ mRNA elevation. Correlations were only performed within pre-mutation carriers of FraX, since our interest was to investigate regions shown to differ between pre-mutation carriers of FraX and controls.

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**Results**

Twenty male pre-mutation carriers of FraX recruited on the basis of genetic, not clinical, phenotype and 20 male controls who did not differ significantly in age, IQ and handedness (Table 1A) participated in the investigation. Genetic testing of pre-mutation carriers provided CGG trinucleotide repeat expansion number, %FMRP(+) lymphocytes and $FMR1$ mRNA levels (Table 1B). While many of the pre-mutation carriers of FraX fell within what is considered a normal range for % FMRP(+), we nonetheless found a significant negative correlation between CGG trinucleotide repeats and %FMRP (+) lymphocytes; as the number of CGG trinucleotide repeats increased, %FMRP(+) lymphocytes was reduced (Kendalls tau = –0.547, $P = 0.002$) (Fig. 1).

Group differences in regional brain anatomy between pre-mutation carriers of FraX and controls were investigated. There were no regions that had significantly greater voxel density for pre-mutation carriers of FraX. However, pre-mutation carriers of FraX had significantly ($P < 0.001$) less grey matter voxel density in: the cerebellum, brainstem, amygda-lo-hippocampal complex, caudate and insula bilaterally; the left thalamus and inferior temporal cortex; and the right pre- and postcentral gyri, and inferior parietal cortex extending to the precuneus. There were also significant ($P < 0.001$) reductions in white matter voxel density in: the cerebellum, brainstem, pons, cingulate and genu of the corpus callosum, and the tracts within the frontal and temporal lobes (Fig. 2 and Table 2).

The clusters identified were then correlated with IQ and age. There were no associations of anatomy and IQ. However, the left hippocampus decreased with age to a significantly greater degree for pre-mutation carriers of FraX compared with controls (Fig. 3).

Finally, we investigated whether, in pre-mutation carriers of FraX, differences in grey and white matter voxel density were related to expansion of CGG trinucleotide repeat number, reduction of %FMRP(+) lymphocytes and/or $FMR1$

### Table 1 Description of subjects

(A) Group matching

<table>
<thead>
<tr>
<th></th>
<th>Carriers mean ± SD, range</th>
<th>Controls mean ± SD, range</th>
<th>$t$ tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (in years)</td>
<td>53.3 ± 14.9, 20–72</td>
<td>47.7 ± 15.3, 18–70</td>
<td>$t = 1.163$ ($P = 0.252$)</td>
</tr>
<tr>
<td>Full-scale IQ (WAIS)</td>
<td>113.3 ± 12.4, 96–142</td>
<td>116.8 ± 19.1, 95–150</td>
<td>$t = -0.691$ ($P = 0.494$)</td>
</tr>
<tr>
<td>Handedness</td>
<td>17 right, 3 left</td>
<td>17 right, 3 left</td>
<td></td>
</tr>
</tbody>
</table>

(B) Genetic variables for male pre-mutation carriers

<table>
<thead>
<tr>
<th>Genetic variable</th>
<th>Mean ± SD, range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGG repeat number</td>
<td>85.6 ± 18.4, 55–137</td>
</tr>
<tr>
<td>%FMRP(+) lymphocytes</td>
<td>75.4 ± 5.4, 68–84</td>
</tr>
<tr>
<td>$FMR1$ mRNA level</td>
<td>3.7 ± 1.6, 1.7–8.0</td>
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</table>

WAIS = Wechsler Adult Intelligence Scale. CGG trinucleotide repeat numbers were also measured for controls; the range was 19–41, the mean 28.68, and SD 5.1 (also see Fig. 1).
mRNA elevation (Table 2 and Fig. 4). There were no associations with FMR1 mRNA elevation. Significant (\( P < 0.01 \)) negative correlations between CGG trinucleotide repeat expansion and grey matter voxel density were observed in the cerebellum, amygdalo-hippocampal complex and insula bilaterally; the left thalamus; and the right caudate, pre- and postcentral gyri, and inferior parietal cortex extending to precuneus. Also, significant (\( P < 0.01 \)) negative correlations between CGG trinucleotide repeat expansion and white matter voxel density were observed in the genu of the corpus callosum, the cingulate, and the tracts within the frontal and temporal lobes bilaterally; and in the right cerebellum. Finally, significant (\( P < 0.01 \)) positive correlations between %FMRP(+) lymphocytes and grey matter voxel density were observed in amygdalo-hippocampal complex bilaterally, the left thalamus and brainstem. We found no significant relationship between any white matter voxel density and %FMRP(+) lymphocytes.

Voxel density in the amygdalo-hippocampal complex bilaterally and the left thalamus was significantly reduced in pre-mutation carriers compared with controls, and within these regions grey matter voxel density was significantly related to CGG trinucleotide repeat expansion and reduction in %FMRP expression (Fig. 5).

**Discussion**

Our molecular genetic investigation showed a significant negative correlation between CGG trinucleotide repeats and %FMRP(+) lymphocytes, replicating previous studies (Tassone et al., 2000b; Kenneson et al., 2001). As the number
of CGG trinucleotide repeats increased, %FMRP(+) lymphocytes was reduced. We did not demonstrate significant correlations between FMR1 mRNA levels and CGG trinucleotide repeats or %FMRP(+) lymphocytes. However, previous studies over a broader range of pre-mutation CGG trinucleotide repeat sizes did observe a positive correlation between CGG trinucleotide repeat size and reduction in %FMRP(+) lymphocytes. However, both of these possibilities seem unlikely because DNA studies of brains of those with pre-mutation FraX alleles in blood have demonstrated consistency between the CGG trinucleotide repeat sizes of those two tissues (Tassone et al., 1999). Further, if pre- and full mutation expression in the brain was random, we would not expect to find an overlap of between-group differences in brain anatomy and within-group genetic variables.

During embryonic development, high levels of FMR1 expression are evident in cerebellum, hippocampus, thalamus and the cerebral cortex in vitro (Abitbol et al., 1993; Hinds et al., 1993). We previously have demonstrated in vivo that female pre-mutation carriers have structural and metabolic abnormalities in these regions (Murphy et al., 1999). Here, we report similar effects in the same brain regions of male pre-mutation carriers, larger CGG trinucleotide repeat size and reduction in %FMRP(+) lymphocytes were associated with less voxel density of the amygdalo-hippocampal complex bilaterally, and the left thalamus. These data provide the first evidence that there is a relationship between age, pre-mutation CGG trinucleotide repeat expansion, %FMRP(+) lymphocytes and neuroanatomy in male pre-mutation carriers of FraX.

Since the pre-mutation carriers in our study were identified by DNA analysis on peripheral blood leukocytes, our results could be explained by: (i) the random presence of pre- and full mutation alleles in regions of the brain, and/or (ii) undetected differences in blood and brain mutation status. However, both of these possibilities seem unlikely because DNA studies of brains of those with pre-mutation FraX alleles in blood have demonstrated consistency between the CGG trinucleotide repeat sizes of those two tissues (Tassone et al., 1999). Further, if pre- and full mutation expression in the brain was random, we would not expect to find an overlap of between-group differences in brain anatomy and within-group genetic variables.

<table>
<thead>
<tr>
<th>Table 2 Significant decreases in regional brain volume for male pre-mutation FraX carriers compared with controls</th>
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<tbody>
<tr>
<td>Talarich and Tournoux coordinates, cluster centroid (size)</td>
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</table>

<table>
<thead>
<tr>
<th>Grey matter differences</th>
<th>Talarich and Tournoux coordinates, cluster centroid (size)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bilateral amygdalo-hippocampal complex</strong></td>
<td>$-30 - 18 - 26 (63)$</td>
</tr>
<tr>
<td>$-22 - 26 - 06 (4)$</td>
<td></td>
</tr>
<tr>
<td>$+23 - 12 - 33 (75)$</td>
<td></td>
</tr>
<tr>
<td>$-08 - 19 +02 (96)$</td>
<td></td>
</tr>
<tr>
<td><strong>Left thalamus</strong></td>
<td>$+24 - 42 - 21 (147)$</td>
</tr>
<tr>
<td>$-04 - 64 - 27 (617)$</td>
<td></td>
</tr>
<tr>
<td>Brainstem</td>
<td>$-05 - 43 - 31 (52)$</td>
</tr>
<tr>
<td>Left lateral inferior temporal cortex</td>
<td>$-52 - 12 - 32 (28)$</td>
</tr>
<tr>
<td>Right insula</td>
<td>$+40 - 05 +09 (115)$</td>
</tr>
<tr>
<td>Left insula and caudate</td>
<td>$-22 +07 - 08 (254)$</td>
</tr>
<tr>
<td>Right caudate</td>
<td>$+18 +07 - 11 (141)$</td>
</tr>
<tr>
<td>Right pre-postcentral gyri</td>
<td>$+61 +03 +20 (70)$</td>
</tr>
<tr>
<td>Right inferior parietal cortex/precuneus</td>
<td>$+31 - 76 +42 (64)$</td>
</tr>
<tr>
<td>White matter differences</td>
<td>$-23 - 57 - 36 (113)$</td>
</tr>
<tr>
<td>Bilateral cerebellum</td>
<td>$+18 - 37 - 34 (177)$</td>
</tr>
<tr>
<td>and right pons</td>
<td>$-08 +04 +13 (1275)$</td>
</tr>
<tr>
<td>Bilateral frontal white matter</td>
<td>$+36 - 38 +09 (590)$</td>
</tr>
<tr>
<td>genu of the corpus callosum</td>
<td>$-35 - 39 +34 (83)$</td>
</tr>
<tr>
<td>temporal white matter and cingulate</td>
<td>$-16 - 27 - 02 (6)$</td>
</tr>
</tbody>
</table>

Statistical analysis (Bullmore et al., 1999) revealed 13 significant clusters in grey matter and six significant clusters in white matter volumes ($P < 0.001$). Brain regions (column 1) are described by the coordinates of cluster centroids, and cluster size (i.e. number of voxels) in parentheses (column 2). These coordinates correspond to the stereotactic atlas of Talairach and Tournoux (1988) and are reported in the order of x (where + is right and − is left), y (where − is posterior to the anterior commissure (AC) line and + is anterior to the AC line) and z (where − is inferior to the AC − posterior commissure (PC) line and + is superior to the AC–PC line). Brain regions also affected by expansion of CGG trinucleotide repeat number (i.e. a negative correlation) and reduction of %FMRP(+) expression (i.e. a positive correlation) are marked * and #, respectively, in column 1. Regions showing an overlap of between-group differences, within-group CGG trinucleotide repeat and %FMRP(+) lymphocytes status are shown in bold. Note that, in white matter, only the right cerebellum was affected by expansion of the CGG trinucleotide repeat number.

LEF T HIPPOCAMPUS

**Carriers:** $r = 0.56$, $P = 0.01$

**Controls:** $r = 0.08$, $P = 0.76$

Zobs = -2.07

**Fig. 3** Correlation of neuroanatomy and age in the left hippocampus presented on a scatterplot showing lines of best fit. Male pre-mutation carriers of FraX are represented by unfilled squares; carriers are represented by filled triangles. Pearson’s r values and statistical significance levels (P) are given for each group, and the zobs value showing a significant difference between the groups.
pre-mutation carriers. This finding is consistent with growing evidence that CGG trinucleotide repeats have biological effects, and may modulate cellular ageing. The molecular data indicate consistently elevated levels of FMR1 mRNA (Tassone et al., 2000b,c; Hagerman et al., 2001; Kenneson et al., 2001), and reduced FMRP production at the higher end (>100 CGG trinucleotide repeats) of the pre-mutation range (Tassone et al., 2000a,b; Kenneson et al., 2001). Some pre-mutation carriers of FraX display perturbation of executive function (Hagerman et al., 2001) and some of the physical features of full mutation FraX (Hagerman, 1999; Tassone et al., 2000a); a subgroup of older pre-mutation males have neurological abnormalities (including tremor, ataxia and brain atrophy) (Hagerman et al., 2001; Brunberg et al., 2002); and there is a high incidence of premature ovarian failure in females (Allingham-Hawkins et al., 1999). Also, the brain regions we report as abnormal in pre-mutation carriers of FraX are crucial to motor and cognitive functions such as coordination and attention, and so they may form the biological substrate for the progressive action tremor, locomotor abnormalities and executive function deficits (Hagerman et al., 2001) reported in the syndrome.

FMRP is an RNA-binding protein (Siomi et al., 1993), required for normal synaptic maturation (Weiler et al., 1997). People with the full mutation of FraX, who have an absence of FMRP, have immature cerebral cortical spine morphology and increased dendritic spine densities (Hinton et al., 1991; Irwin et al., 2000). Further, FMR1 knockout

Fig. 4 Correlations of neuroanatomy and genetic variables for (A) grey and (B) white matter differences correlating negatively with CGG trinucleotide repeat size, and (C) grey matter differences correlating positively with %FMRP(+) lymphocytes. Regions shown in yellow are those that are significantly correlated with CGG trinucleotide repeat expansion and/or reduction in %FMRP(+) lymphocytes within the group of pre-mutation carriers.
a. Grey Matter Decreases Correlated with Increases in CGG Repeat Size

![Graphs showing negative correlations between grey matter decreases and CGG repeat size.](image)

Negative correlations of grey matter decreases with CGG trinucleotide repeat size shown in yellow, on axial brain slices. Scatterplots illustrate the correlations with lines of best fit. Left amygdala–hippocampal complex, Kendall’s tau = −0.586 (P = 0.001); right amygdala–hippocampal complex, Kendall’s tau = −0.444 (P = 0.009); left thalamus, Kendall’s tau = −0.538 (P = 0.001).

b. Grey Matter Decreases Correlated with Decreases in %FMRP(+) Lymphocytes

![Graphs showing positive correlations between grey matter decreases and %FMRP(+) lymphocytes.](image)

Positive correlations of grey matter decreases with %FMRP(+) lymphocytes, shown in yellow, are illustrated on axial brain slices and scatterplots. Left amygdala–hippocampal complex, Kendall’s tau = 0.629 (P = 0.000); right amygdala–hippocampal complex, Kendall’s tau = 0.535 (P = 0.002); thalamus, Kendall’s tau = 0.656 (P = 0.000). Note that (A) and (B) portray separate analyses: (A) correlation of grey matter volume and CGG trinucleotide repeat number, (B) correlation of grey matter volume and %FMRP(+) lymphocytes, and thus result in different anatomical clusters within the same anatomical regions.

Fig. 5 (A) Negative correlations of grey matter decreases with CGG trinucleotide repeat size shown in yellow, on axial brain slices. Scatterplots illustrate the correlations with lines of best fit. Left amygdala–hippocampal complex, Kendall’s tau = −0.586 (P = 0.001); right amygdala–hippocampal complex, Kendall’s tau = −0.444 (P = 0.009); left thalamus, Kendall’s tau = −0.538 (P = 0.001). (B) Positive correlations of grey matter decreases with %FMRP(+) lymphocytes, shown in yellow, are illustrated on axial brain slices and scatterplots. Left amygdala–hippocampal complex, Kendall’s tau = 0.629 (P = 0.000); right amygdala–hippocampal complex, Kendall’s tau = 0.535 (P = 0.002); thalamus, Kendall’s tau = 0.656 (P = 0.000). Note that (A) and (B) portray separate analyses: (A) correlation of grey matter volume and CGG trinucleotide repeat number, (B) correlation of grey matter volume and %FMRP(+) lymphocytes, and thus result in different anatomical clusters within the same anatomical regions.
mice have abnormal dendritic spines (Irwin et al., 2002), hyperactivity and deficits in learning and memory (Dutch–Belgian Fragile X Consortium, 1994); and recent studies of Drosophila mutant flies (dfxr) have demonstrated synaptic alterations and impaired circadian rhythms (Gao, 2002). Overexpression of FMR1 homologues in mice and flies produces opposing adverse effects, such as lethargy (Bardoni and Mandel, 2002). Although little is known about the effects of reduced FMRP production and elevated FMR1 mRNA levels on the human brain, post-mortem of four pre-mutation male carriers of FraX (who were affected with the tremor/ataxia syndrome) revealed spherical eosinophilic intranuclear inclusion bodies in neurons and glial cells (Greco et al., 2002). Further, a functional neuroimaging study of females with full mutation FraX alleles found correlations of FMRP production and brain activity in frontal and supramarginal cortices during a working memory task (Kwon et al., 2001). Thus, not only is production of FMRP essential for brain maturation, but variations in FMRP production may also affect brain anatomy and function.

Conclusions

The results of this study reveal, for the first time, that there is a relationship between ageing, pre-mutation CGG trinucleotide repeat expansion, %FMRP(+) lymphocytes and brain anatomy in pre-mutation carriers of FraX recruited on the basis of genetic, not clinical, phenotype. These findings may provide the neuroanatomical basis for previously reported clinical effects of pre-mutation expansion of CGG trinucleotide repeats. This is of relevance to the general population, since ~1 : 813 men are pre-mutation carriers of FraX with >54 CGG trinucleotide repeats (Dombrowski et al., 2002), with the ratio closer to 1 : 100 for women (Hagerman and Hagerman, 2002); and ~1 : 208 are pre-mutation carriers of FraX with 50–200 CGG trinucleotide repeats (Youngs et al., 2000). Our findings may also be pertinent to the understanding of other neuropsychiatric disorders associated with expansion of trinucleotide repeats.

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