ORIGINAL ARTICLE

Brain structure and intragenic DNA methylation are correlated, and predict executive dysfunction in fragile X premutation females

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DNA methylation of the Fragile X mental retardation 1 (FMR1) exon 1/intron 1 boundary has been associated with executive dysfunction in female carriers of a FMR1 premutation (PM: 55–199 CGG repeats), whereas neuroanatomical changes have been associated with executive dysfunction in PM males. To our knowledge, this study for the first time examined the inter-relationships between executive function, neuroanatomical structure and molecular measures (DNA methylation and FMR1 mRNA levels in blood) in PM and control (< 44 CGG repeats) females. In the PM group, FMR1 intron 1 methylation was positively associated with executive function and cortical thickness in middle and superior frontal gyri, and left inferior parietal gyrus. By contrast, in the control group, FMR1 intron 1 methylation was negatively associated with cortical thickness of the left middle frontal gyrus and superior frontal gyri. No significant associations were revealed for either group between FMR1 mRNA and neuroanatomical structure or executive function. In the PM group, the lack of any significant association between FMR1 mRNA levels and phenotypic measures found in this study suggests that either FMR1 expression is not well conserved between tissues, or that FMR1 intron 1 methylation is linked to neuroanatomical and cognitive phenotype in PM females via a different mechanism.

INTRODUCTION

Trinucleotide CGG repeat expansions of the Fragile X mental retardation 1 (FMR1) gene are related to a number of Fragile X-associated disorders. Full mutation alleles (FM: greater than 200 CGG repeats) are associated with silencing of FMR1 through methylation of the promoter region located in the 5′ untranslated region,1 resulting in a neurodevelopmental disorder known as Fragile X syndrome. The prevalence of Fragile X syndrome in the general population is ~1 in 4000.2 The more common FMR1 premutation (PM) expansion (55–199 CGG repeats), which is found in ~1 in 209 females and 1 in 430 males,3 confers the risk of developing Fragile X-associated tremor/ataxia syndrome (FXTAS). FXTAS is a progressive neurodegenerative disorder, thought to result in, part, from elevated levels of FMR1 mRNA, leading to protein aggregation (ubiquitin-positive intracellular inclusion bodies likely due to repeat-associated non-AUG-initiated translation) and reduced neuronal cell function.4–6 FXTAS manifests in a range of neurological and clinical symptoms as well as executive dysfunction.7 Executive dysfunction, specifically pertaining to working memory and response inhibition processes, has been reported in both PM males8–10 and females without FXTAS,11–15 and may represent either an independent PM phenotype or a precursor to FXTAS.

Significant associations between neuroanatomical structure (white and grey matter) and measures of cognition, including executive function, have been reported in PM males and females.16–21 More recently, a link has also been demonstrated between molecular changes and the risk of developing executive dysfunction in PM females; specifically, methylation changes at the FMR1 exon 1/intron 1 boundary measured in blood DNA—a region also known as Fragile X-related epigenetic element 2 (FREE2).22 To our knowledge, this study for the first time examined whether CGG repeat length, FMR1 mRNA levels and methylation levels of the CpG island (or the activation ratio, AR) and FREE2 region correlate significantly with altered neuroanatomy in PM females without FXTAS. It also examined the relationships between these molecular and neural measures and cognitive performance; specifically, changes in executive function based on an ocular motor switch task.

MATERIALS AND METHODS

Participants

CGG repeat lengths were determined for 36 females aged between 22 and 54 years. Of these, 19 exhibited PM alleles with a CGG repeat length between 55 and 199, and 17 exhibited normal alleles with CGG repeat length < 44 (thus providing control data). All were recruited from support groups and population-based Fragile X carrier screening studies,23 as well as local networks and via online advertisements.

All participants were English-speaking, had normal (or corrected) vision and hearing, and had no history of any serious neurological damage/disease (including FXTAS). Exclusion criteria extended to those who thought they may be pregnant, as well as with any...
magnetic resonance imaging (MRI) contraindication. Ethics approval for this study was granted by the Monash University and Southern Health Human Research Committees (Project Number 10147B); all participants gave their informed consent before inclusion in the study in accordance with the Declaration of Helsinki.

Molecular analyses
DNA was extracted from whole blood for CGG sizing and methylation analysis. The AmpliDex FMR1 PCR Kit was used for CGG sizing, as per the manufacturer’s instructions (Asuragen, Austin, TX, USA). RNA was extracted from peripheral blood mononuclear cells, followed by cDNA synthesis and real-time PCR gene expression analysis performed on a Viia 7 Real-Time PCR System (Life Technologies, Global). The relative standard curve method was utilised for FMR1 S′ and 3′ mRNA quantification normalised to mRNA levels of two internal control genes (SDHA and EFTAA2), as previously described. AR was determined using methylation-sensitive Southern blot targeting a Nrhl restriction site within the FMR1 CpG island, as previously described. The Epityper system was used to analyse FREE2 methylation in the blood, consisting of five CpG unit outputs (targeting nine CpG sites) per sample tested. Blood DNA from each participant was bisulphite-converted in duplicate, with each conversion analysed twice using the Epityper system. A summary measure for each CpG unit was determined as the mean of the four methylation output ratio measurements per sample. These procedures resulted in a total of eight molecular measures: CGG, AR, FMR1 mRNA, FMR1 exon 1 (CpG 1 and CpG 2) and intron 1 (CpG 6/7, CpG 8/9 and CpG 10–12) methylation markers.

Assessment and analysis of executive function
Haylings Sentence Completion Test. The Haylings Sentence Completion Test, a test of response inhibition, required participants to respond to 15 sentences with the last word omitted, by providing a word that was unconnected to the sentence. Responses were classified as either correct, a Category A error (word plausibly finished the sentence) or Category B error (word was somewhat connected to the sentence)—both of which measure inhibitory processing. The total number of Category A and Category B errors were recorded, with larger error numbers indicating impaired response inhibition processes.

Ocular motor switch task. The ocular motor switch task assesses attention, response inhibition and working memory processes. It required participants to move their eye either towards (prosaccade trial) or away (antisaccade) from a target as quickly and as accurately as possible depending on a central colour cue given at the start of each trial (Supplementary Note 1 for more details). As this study was interested in executive dysfunction, antisaccade data were removed from this analysis to avoid any contamination of the paradoxical ‘benefit’ that is commonly seen for antisaccade trials following a prosaccade trial (antisaccade switch trials). This yielded a total of seven prosaccade variables: correct latency (ms), error latency (ms), time to correct (ms), switch/non-switch directional error percentage and switch/non-switch anticipatory error percentage.

MRI acquisition and analysis
Structural MRIs were acquired on a 3 T Siemens Magneto Skyra scanner using a 20-channel head coil using a T1-weighted three-dimensional MP-RAGE scan (208 sagittal slices of 1 mm thickness (no gap), repetition time = 1540 ms, echo time = 2.55 ms, inversion time = 900 ms, a flip angle of 9°, field of view = 256 × 256 mm², yielding a standard voxel size = 1 x 1 x 1 mm³). T1-weighted three-dimensional MP-RAGE data were analysed using FreeSurfer version 5.1.0 (http://surfer.nmr.mgh.harvard.edu) with technical details previously described. Automated anatomic segmentation procedure was used to measure volume of T1 white matter hypointensities whereas regional cortical thickness measures were obtained from the automated anatomic parcellation procedure for each participant.

Regional cortical thickness from the middle and superior frontal gyri (representing the dorsolateral prefrontal cortex) and inferior parietal gyrus from both left and right hemispheres were selected as they are pivotally involved in the control of saccades.
The differences in the relationships between methylation markers CpG 6/7 and CpG 8/9 and cortical thickness between PM and control females suggest that in normal neurobiology, FMR1 methylation (potentially X chromosome inactivation (XCI)) is related to thickness of specific cortical regions and volume of white matter hypointensities, which are disrupted in PM females without FXTAS through a currently unknown mechanism that modifies the observed associations.

**DISCUSSION**

Understanding the disorder-specific role of intragenic DNA methylation is critically important, providing a unique opportunity to investigate gene/environment interactions of clinical significance. In this study, highly significant relationships were found between the intragenic methylation within the 5′ end of the FMR1 intron 1 and phenotype measures of executive function, volume of white matter hypointensities and regional cortical thickness in the frontal and parietal cortices of PM females without FXTAS. The differences in the relationships between methylation markers CpG 6/7 and CpG 8/9 and cortical thickness between PM and control females suggest that in normal neurobiology, FMR1 methylation (potentially X chromosome inactivation (XCI)) is related to thickness of specific cortical regions and volume of white matter hypointensities, which are disrupted in PM females without FXTAS through a currently unknown mechanism that modifies the observed associations.

**FMR1 intron 1 methylation, but not FMR1 mRNA, predicts executive dysfunction in PM females**

In PM females without FXTAS, decreased methylation of both FMR1 promoter (AR) and FMR1 intron 1 regions was found to relate to executive dysfunction. This relationship was absent in controls entirely. Further, the strongest relationships for each composite cognitive score were seen within the 5′ end of FMR1 intron 1, as compared with methylation of exon 1 or AR. This is consistent with the study by Cornish and colleagues, supporting the prior hypothesis that methylation of FMR1 intron 1 CpG sites is a good predictor of deficits within the executive function phenotype of PM and FM females. Unlike previous ocular motor studies, FMR1 mRNA levels were not correlated with executive function scores in this cohort of PM females without FXTAS. Conversely, FMR1 intron 1 methylation correlated with both executive function and neuroanatomical structure in the PM group. We also found no significant relationships between any methylation measure (AR and FREE2 methylation) and neuroanatomical relationships were found for the control group (Figure 1c, Figure 2 and Table 2). Interaction analysis revealed that significant group differences in the relationships between FMR1 intron 1 methylation and middle frontal, superior frontal and inferior parietal thickness were evident (Table 3).

Neuroanatomical measures were related to executive function measures for both PM and control groups. The three significant relationships for the PM group suggest that executive dysfunction deficits, denoted by composite cognitive scores, were related to increased white matter hypointensities (prosaccade response time: coefficient ($\beta$) = 0.491, s.e. = 0.21, $P = 0.033$, $r^2 = 0.241$) and decreased cortical thickness in frontal lobe regions. No significant CpG 8/9–neuroanatomical relationships were found for the control group (Figure 1c, Figure 2 and Table 2). Interaction analysis revealed that significant group differences in the relationships of the FMR1 intron 1 and phenotype measures of executive function, volume of white matter hypointensities and regional cortical thickness in the frontal and parietal cortices of PM females without FXTAS. The differences in the relationships between methylation markers CpG 6/7 and CpG 8/9 and cortical thickness between PM and control females suggest that in normal neurobiology, FMR1 methylation (potentially X chromosome inactivation (XCI)) is related to thickness of specific cortical regions and volume of white matter hypointensities, which are disrupted in PM females without FXTAS through a currently unknown mechanism that modifies the observed associations.
Table 1. Relationships between molecular parameters and composite cognitive scores and neuroanatomical measures outcome variables for the PM group

| Outcome variables                      | CGG    | AR      | FMR1 mRNA | CpG 1   | CpG 2   | CpG 6/7 | CpG 8/9 | CpG 10–12 |
|----------------------------------------|--------|---------|------------|---------|---------|---------|---------|-----------|
| Prosaccade response time               | −0.142 (0.020) | 0.450 (0.199) | 0.288 (0.093) | −0.105 (0.011) | 0.232 (0.054) | 0.791 (0.366) | 0.321 (0.103) | 0.281 (0.064) |
| Prosaccade error score                 | 0.217 (0.047) | −0.469 (0.350) | 0.038 (0.001) | 0.364 (0.261) | −0.253 (0.064) | −0.76 (0.353) | −0.591 (0.344) | −0.67 (0.478) |
| Executive function score               | 0.229 (0.053) | −0.196 (0.009) | 0.132 (0.117) | 0.010 (0.000) | 0.213 (0.045) | −0.455 (0.198) | −0.511 (0.261) | −0.310 (0.086) |
| White matter hypointensities           | −0.079 (0.006) | 0.386 (0.158) | 0.265 (0.100) | −0.511 (0.261) | 0.113 (0.013) | 0.471 (0.222) | 0.076 (0.005) | 0.405 (0.079) |
| Left middle frontal gyrus              | −0.146 (0.021) | 0.215 (0.051) | −0.254 (0.066) | 0.106 (0.011) | 0.331 (0.109) | 0.427 (0.179) | 0.564 (0.319) | 0.504 (0.220) |
| Right middle frontal gyrus             | 0.035 (0.001) | 0.039 (0.002) | −0.346 (0.16) | 0.065 (0.004) | 0.055 (0.003) | 0.250 (0.062) | 0.655 (0.430) | 0.375 (0.117) |
| Left superior frontal gyrus            | −0.024 (0.001) | −0.051 (0.003) | −0.324 (0.104) | 0.252 (0.064) | 0.292 (0.085) | 0.426 (0.176) | 0.541 (0.293) | 0.455 (0.183) |
| Right superior frontal gyrus           | 0.030 (0.001) | 0.065 (0.005) | −0.186 (0.033) | 0.305 (0.093) | 0.276 (0.076) | 0.293 (0.086) | 0.662 (0.374) | 0.415 (0.168) |
| Left inferior parietal gyrus           | −0.081 (0.007) | −0.200 (0.040) | −0.294 (0.084) | 0.058 (0.003) | 0.104 (0.011) | 0.558 (0.312) | 0.494 (0.241) | 0.317 (0.077) |
| Right inferior parietal gyrus          | −0.020 (0.000) | −0.242 (0.056) | −0.350 (0.119) | −0.039 (0.002) | 0.052 (0.003) | 0.242 (0.045) | 0.089 (0.008) | −0.023 (0.000) |

Abbreviations: β, standardized regression coefficients; AR, activation ratio; FMR1, fragile X mental retardation 1; PM, premutation; r², coefficient of determination. Note: Figures in bold indicate that P < 0.05.

Table 2. Relationships between molecular parameters and composite cognitive scores and neuroanatomical measures outcome variables for the healthy control group

| Outcome variables                      | CGG    | AR      | FMR1 mRNA | CpG 1   | CpG 2   | CpG 6/7 | CpG 8/9 | CpG 10–12 |
|----------------------------------------|--------|---------|------------|---------|---------|---------|---------|-----------|
| Prosaccade response time               | −0.249 (0.062) | −0.353 (0.125) | 0.475 (0.225) | 0.148 (0.020) | −0.267 (0.067) | 0.087 (0.008) | 0.075 (0.006) | −0.054 (0.003) |
| Prosaccade error score                 | 0.109 (0.018) | −0.011 (0.000) | −0.282 (0.190) | 0.032 (0.002) | 0.296 (0.159) | 0.314 (0.099) | 0.153 (0.023) | 0.205 (0.042) |
| Executive function score               | 0.201 (0.040) | 0.248 (0.061) | −0.402 (0.162) | 0.152 (0.023) | 0.089 (0.009) | 0.016 (0.000) | −0.330 (0.109) | −0.164 (0.027) |
| White matter hypointensities           | −0.426 (0.206) | −0.254 (0.074) | 0.360 (0.130) | 0.105 (0.011) | 0.271 (0.071) | 0.418 (0.174) | 0.429 (0.184) | 0.206 (0.042) |
| Left middle frontal gyrus              | 0.117 (0.010) | −0.271 (0.054) | −0.158 (0.025) | −0.015 (0.000) | −0.588 (0.334) | −0.651 (0.424) | −0.466 (0.217) | −0.621 (0.386) |
| Right middle frontal gyrus             | 0.133 (0.014) | −0.280 (0.078) | −0.084 (0.006) | −0.124 (0.015) | −0.151 (0.021) | −0.293 (0.086) | −0.045 (0.002) | −0.261 (0.068) |
| Left superior frontal gyrus            | −0.454 (0.063) | −0.212 (0.045) | −0.306 (0.093) | 0.253 (0.030) | −0.022 (0.000) | −0.378 (0.143) | −0.119 (0.014) | −0.728 (0.302) |
| Right superior frontal gyrus           | 0.164 (0.021) | −0.297 (0.088) | −0.259 (0.067) | −0.076 (0.005) | −0.085 (0.007) | −0.507 (0.257) | −0.175 (0.031) | −0.521 (0.166) |
| Left inferior parietal gyrus           | 0.098 (0.008) | −0.269 (0.072) | −0.293 (0.086) | −0.349 (0.132) | −0.006 (0.000) | −0.426 (0.182) | −0.076 (0.006) | −0.302 (0.079) |
| Right inferior parietal gyrus          | −0.433 (0.056) | −0.052 (0.002) | −0.340 (0.115) | −0.081 (0.000) | 0.060 (0.004) | −0.043 (0.002) | −0.310 (0.087) | −0.301 (0.078) |

Abbreviations: β, standardized regression coefficients; AR, activation ratio; FMR1, fragile X mental retardation 1; r², coefficient of determination. Note: Figures in bold indicate that P < 0.05.
methylation) and FMR1 mRNA for PM or control groups. This suggests that, in PM females without FXTAS, FMR1 intron 1 methylation has clinical significance involving a different mode or pathway of action that does not directly involve overexpression of FMR1 mRNA.

It is important to note that in this study FMR1 mRNA was normalised to two control genes (SDHA and EIF4A2) and not beta-glucuronidase (GUS), as in previous observations assessing differing aspects of executive function.22,48,49 GUS is a commonly used reference gene or internal control for transcript quantification with PCR. In a study of FM males where FMR1 mRNA was normalised to actin B and GUS, a positive linear relationship between FMR1 mRNA and methylation of the FMR1 promotor region was found,50 which was not evident in this study. This difference could have several explanations including that (a) the Brasa et al. study50 performed correlation analyses for different CpG sites, (b) used FM males as opposed to PM females without FXTAS, (c) had a much smaller sample size of only seven individuals (susceptible to the effects of outliers) or most likely (d) used a different normalisation strategy of FMR1 mRNA. In relation to the last potential explanation, it is important to note that variability in gene expression of internal control genes has been well documented to have an impact on target gene real-time PCR outputs,51 which we have recently shown to apply in PM females without FXTAS.52

FMR1 intron 1 differently predicts neuroanatomical structure between PM and control groups

Juxtaposing associations were found between increased FREE2 methylation and cortical thickness in our PM and control groups: increased cortical thickness for the PM group and decreased cortical thickness for the control group. This was most evidenced when assessing the FREE2 methylation relationships with cortical thickness of the left middle frontal gyrus, where there was a trend towards increased cortical thickness for the PM group compared with controls (P = 0.058). The clear dissociation between FMR1 intron 1 methylation and cortical thickness of the left middle frontal gyrus, as well as other FMR1 intron 1 methylation–frontal and inferior parietal relationships, between groups, suggests a possible involvement for XCI skewing in regulating the thickness...
Multiple neuroanatomical correlates of executive function found in the PM group

Each of the composite cognitive scores was found to be associated with either regional cortical thickness or volume of white matter hypointensities within the frontoparietal executive processing network (Model III) for PM females without FXTAS, whereas only inferior parietal thickness related to the prosaccade error score in the control group. Specifically, a positive relationship was found between white matter hypointensities and prosaccade reaction time in PM females without FXTAS, which is consistent with the hypothesis that reduced white matter integrity results in increased response times in cognitive tasks generally.63

Similarly to our findings of an association between left middle frontal gyrus thickness and prosaccade error scores, decreased cortical thickness in the middle frontal cortex has been linked to executive dysfunction.59 Equally, we also reveal that decreased cortical thickness of the left inferior parietal gyrus related to impaired executive function scores in PM females without FXTAS. Collectively, these findings are in direct contrast to a previous Fragile X syndrome study, where increased cortical thickness was associated with poorer performance on multiple domains of the Stanford-Binet Intelligence Scale.60 In that study, the Fragile X syndrome findings were hypothesised to reflect inefficient synaptic pruning due to FMRP deficiencies.60 As such, other mechanism(s) and pathways discussed below are likely to underlie these neuroanatomical–executive function relationships in PM females without FXTAS.

Alternative explanations to the observed relationships

The process of XCI, where only one of the two X chromosomes becomes inactivated in females, is complex and relies on a number of factors including DNA methylation, non-coding RNAs and nuclear protein. DNA methylation is an important process in the regulation of XCI and gene expression. DNA hydroxymethylation (5-hydroxymethylcytosine (5hmC)), is thought to be an epigenetic modifier and a possible intermediate product within an active DNA demethylation pathway, potentially having a role in both neurodevelopmental and neurodegenerative diseases/disorders.61–63 In a FXTAS mouse model, 5hmC levels were found to be reduced compared to wild-type littermates, suggesting that for PM individuals, 5hmC may have a neurodegenerative role.64 Moreover, non-coding RNAs are most commonly derived from intragenic DNA regions.65 Specifically, RNA:DNA hybrids are thought to form at the location of FMR1 intron 1 CpG sites39 and may also have a role in XCI. Further, overexpression of ASFMR1 and long non-coding RNA have previously been reported in PM individuals,66 and have also been associated with parkinsonism and mitochondrial dysfunction.67 Future studies should explore the contribution of the aforementioned pathways as alternative explanations for the relationships observed in this study between FMR1 intron 1 methylation and phenotype measures.

Table 3. The interaction effect of group by composite cognitive score and neuroanatomical measures for each molecular parameter

| Outcome variables | F (ηp2) |
|-------------------|---------|
|                  | CpG 1   |
|                  | CpG 2   |
|                  | CpG 8/9 |
|                  | CpG 8/7 |
| Prosaccade reaction time | 2.353 (0.133) |
| Prosaccade error scores | 4.012 (0.025) |
| Executive function score | 6.620 (0.286) |
| White matter hypointensities | 3.633 (0.132) |
| Left middle frontal gyrus thickness | 1.914 (0.055) |
| Right middle frontal gyrus thickness | 1.803 (0.017) |
| Left superior frontal gyrus thickness | 1.712 (0.049) |
| Right superior frontal gyrus thickness | 1.284 (0.017) |
| Left inferior parietal gyrus thickness | 0.962 (0.029) |
| Right inferior parietal gyrus thickness | 0.727 (0.046) |

Abbreviations: AR, activation ratio; F, F-test statistic; ηp2, partial eta squared; FMR1, Fragile X mental retardation 1; PM, Prader-Willi syndrome; 5hmC, 5-hydroxymethylcytosine; XCI, X-chromosome inactivation.
CONCLUSION
Overall, understanding how epigenetic changes influence neuroanatomy, executive function and clinical outcomes is highly important for both FMR1 PM- and FM-related disorders, and broader neurological disorders influenced by abnormal XCI. Although preliminary, this is, to our knowledge, the first study to link FMR1 intron 1 methylation and neuroanatomical structure in PM and control females. Second, FMR1 intron 1 methylation produced the greatest number of associations (for both phenotype measures), compared with FMR1 exon 1 methylation, AR, CGG repeat size and FMR1 mRNA levels in the blood, confirming our previous observation.22 Frontal and parietal cortical thickness, as well as white matter hypointensities, in brain regions that support executive function, also negatively related to composite cognitive scores. Importantly, differences in the relationships between FMR1 intron 1 methylation and left middle frontal gyrus thickness, and between CpG site 8/9 and frontal and parietal cortical thickness, suggest that XCI skewing in controls may be critical when assessing changes in cortical thickness in females with other neurological diseases. Whereas we provide specific hypotheses regarding the mechanisms underlying such relationships, further confirmatory analysis of the molecular pathways that link FMR1 intron 1 methylation to neuroanatomical structure and executive dysfunction are needed to support these assertions for the PM neurocognitive phenotype and in normal neurobiology. Importantly, together with our previous studies, the utility of FREE2 methylation, particularly methylation of the 5′ FMR1 intron 1 region, as a sensitive measure that relates to both neuroanatomical structure and executive dysfunction in PM females without FXTAS, has been now confirmed.

CONFIDENT OF INTEREST
D Golder is an inventor of the following patents, PCT/AU2010/001134; filing no. AU2010/903595; filing no. AU2011/902500; filing no. 2013/900227; related to the technology described in this publication. The remaining authors declare no conflict of interest.

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