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Symbolic sequence learning is associated with cognitive–affective profiles in female *FMR1* premutation carriers

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This study examines implicit sequence learning impairments that may indicate at-risk cerebellar profiles proposed to underlie some aspects of subtle cognitive and affective dysfunctions found among female *fragile X mental retardation 1* (*FMR1*) premutation (PM)-carriers. A total of 34 female PM-carriers and 33 age- and intelligence-matched controls completed an implicit symbolically primed serial reaction time task (SRTT) previously shown to be sensitive to cerebellar involvement. Implicit learning scores indicated a preservation of learning in both groups; however, PM-carriers demonstrated poorer learning through significantly elevated response latencies overall and at each specific block within the symbolic SRTT. Group comparisons also revealed a core deficit in response inhibition, alongside elevated inattentive symptoms in female PM-carriers. Finally, strong and significant associations were observed between poor symbolic SRTT performance and executive, visuospatial and affective deficits in the PM-carrier group. These associations remained strong even after controlling motor speed, and were not observed in age- and intelligence quotient-matched participants. The findings implicate cerebellar non-motor networks subserving the implicit sequencing of responses in cognitive–affective phenotypes previously observed in female PM-carriers. We contend that symbolic SRTT performance may offer clinical utility in future pharmaceutical interventions in female PM-carriers.

Keywords: Cerebellar cognitive affective syndrome, cortico-cerebellar networks, *FMR1* gene, FMRP, FXTAS, phenotype, premutation carrier

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Fragile X syndrome (FXS) is the most common single gene cause of intellectual disability and autism and is caused by an expanded CGG-repeat sequence (>200) on the *fragile X mental retardation 1* (*FMR1*) gene. Approximately 1 in 209 females carry a premutation (PM) expansion (CGG: 55-200) that can result in a deleterious phenotype through elevated levels of *FMR1* mRNA and reduced fragile X mental retardation protein (FMRP) (Tassone *et al.* 2000, 2012). Females with the PM allele are at increased risk for developing a late onset neurodegenerative disorder, fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman *et al.* 2001). Although there are some protective effects of the second X chromosome, recent studies have documented subtle motor and postural control abnormalities, alongside poor response inhibition and psychiatric dysfunction (Kraan *et al.* 2013a). Given more recent emphasis on the involvement of the cerebellum in higher order cognitive functions, it remains to be determined the extent of influence of *FMR1* gene expansion on subtle cognitive and affective manifestations.

Recent studies in young adult female carriers have provided evidence to suggest a role of the cerebellum in motor and balance impairments (Chonchaiya *et al.* 2010; Goodrich-Hunsaker *et al.* 2011b; Kraan *et al.* 2013b; Narcisa *et al.* 2011); motor sequencing deficits (Loesch *et al.* 2003); and subtle impairments in response inhibition (Kraan *et al.* 2013c), visuospatial processing (Goodrich-Hunsaker *et al.* 2011a; Keri & Benedek 2009, 2010) and psychiatric functioning (Lachiewicz *et al.* 2010; Roberts *et al.* 2009). This is consistent with the features of cerebellar cognitive affective syndrome (CCAS), which is characterized by a range of impairments in executive, visuospatial and affective functions arising from disrupted cortico-cerebellar connectivity (Molinari & Leggio 2013; Schmahmann 2004; Tedesco *et al.* 2011).

Symbolic sequence learning may provide a sensitive paradigm to explore the role of the cerebellum in these

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Table 1: Means and standard deviations of sample characteristics for female *FMR1* PM-carriers and control participants

	FMR1 PM-carriers ($N = 34$) M ± SD (range)	Controls ($N = 33$) M ± SD (range)	*t*-Value	*P*-value
Age (years)	40.88 ± 8.32 (22–55)	41.18 ± 8.18 (22–55)	−0.149	0.882
FSIQ	110.61 ± 10.92 (88–128)	113.00 ± 7.83 (89–129)	−1.023	0.310
VIQ	107.06 ± 13.84 (73–128)	108.70 ± 10.86 (88–136)	−0.534	0.595
PIQ	111.00 ± 11.27 (87–133)	115.27 ± 9.40 (93–133)	−1.682	0.097
CGG-repeat length	86 ± 15.14 (59–122)	31 ± 3.63 (20–42)	19.98	<0.001**
Socioeconomic disadvantage (% < AUD 51 999)	8.8%	15.2%		0.785†
Use of psychotropic medications (% yes)	17.6%	9.1%		0.305†
Child special needs (% yes)	64.7%	18.2%		<0.001†,**

PIQ, performance intelligence quotient; SD, standard deviation; VIQ, verbal intelligence quotient.
† χ^2 test for independence.
** $P < 0.001$.

at-risk profiles in the female PM. It is thought that the cerebellum is important for associating the 'stimulus' with the 'response' command (Bo *et al.* 2011b; Spencer & Ivry 2009). This is consistent with inverse internal models that propose that cerebellar predictions are compared with actual feedback and performance error is adjusted accordingly (Ito 2008; Koziol *et al.* 2010). The imaging evidence for dysfunction along cortico-cerebellar circuitry (middle cerebellar peduncles) in asymptomatic male PM-carriers (Battistella *et al.* 2013; Hashimoto *et al.* 2011; Wang *et al.* 2013), alongside increased activation of the cerebellum during symbolic sequence learning (Bo *et al.* 2011a,b), raises the possibility of a cerebellar role in the previously reported cognitive and affective profiles in the female PM.

Here, we examine symbolic sequence learning to explore the ability to acquire and flexibly adapt to symbolic cue information, and its association with 'signature' female PM weaknesses in executive function, visuospatial processing and psychological symptoms. We predict that female poor symbolic sequence learning will be related to cognitive and affective manifestations associated with expansion of the *FMR1* gene.

Methods

Participants

A total of 35 females with the PM (hereafter named PM-carriers) aged between 22 and 55 were recruited through support groups and population-based fragile X carrier screening studies (Martyn *et al.* 2013; Metcalfe *et al.* 2008). The specific announcement was entitled, 'An Australian study of families who have expansions in the gene associated with fragile X'. A further 35 female control participants aged between 22 and 55 years were also recruited through population-based fragile X carrier screening studies, and through local networks and via online advertisements. The final analysis included 34 PM-carriers and 33 controls owing to three incidences in which the software did not record serial reaction time task (SRTT) performance. All participants were English speaking with no history of epilepsy or of a serious head injury and had normal (or corrected) vision and hearing, and no sign of color blindness or intellectual disability as assessed using the Wechsler Abbreviated Scale of Intelligence [WASI; full scale intelligence quotient (FSIQ) < 70]. The FXTAS rating scale (Leehey 2009) was used to screen all participants for features related to FXTAS – that is, tremor, ataxia or parkinsonism – or any other neuromotor disorder. The comparisons between female PM-carriers and controls showed that the two groups were well matched on age, IQ, socioeconomic status and current use of psychotropic medication(s); however, female PM-carriers were significantly more likely to be caring for a child with special needs ($P = 0.001$). The small portion of medicated women in this study were taking antidepressants at the time of testing, with two female PM-carriers additionally taking antipsychotic medications and one PM-carrier additionally taking a stimulant. All study participants provided signed informed consent and the study procedures were consistent with the Declaration of Helsinki and approved by the Southern Health Ethics Committee (project 10147B). The descriptive demographics and related statistics are shown in Table 1.

Molecular analyses

To ascertain CGG repeat size, DNA was extracted from 2 ml whole blood from all participants using the Promega Maxwell® 16 Instrument and associated Maxwell 16 Blood DNA Purification Kit (Promega Cat No.: AS1010; Promega, Maddison, WI, USA). Polymerase chain reaction (PCR) was performed using the Asuragen® (Asuragen Inc., Austin, TX, USA) AmplideX™ (Life Technologies, Carlsbad, CA, USA) *FMR1* PCR Kit as this assay has been shown to detect a full range of fragile X expanded alleles (Chen *et al.* 2010). The PCR products were assessed via capillary electrophoresis on an Applied Biosystems (Life technologies, Carlsbad, CA, USA) 3730 Genetic Analyzer with electropherogram analysis conducted using GeneMapper® software, version 4.1. All procedures were performed in accordance with the manufacturer's instructions.

Neurobehavioral measures

Implicit sequence learning

A computer-based symbolic SRTT was employed to measure implicit learning. On the basis of previous studies that have shown that non-spatial 'symbolic' cues were most sensitive to cerebellar dysfunctions and functions, we adopted stationary colored circles (red, green, blue and yellow) in favor of the traditional non-colored parallel and spatially located cues used in classic SRTT paradigms (Nissen & Bullemer 1987). Participants were instructed to place middle and index fingers of the left hand on keys 'Z' and 'X', and middle and index fingers of the right hand over keys 'N' and 'M'. Each key corresponded to a circle color (Z: blue; X: red; N: green and M: yellow). On screen, a colored circle was presented. Participants were told to respond by pressing down on the matching key as fast as possible. If the key-press did not correspond to the associated color presented on screen, an 'X' cross over the colored circle prompted the participant to try again. When the key-press was correct, the colored circle stimulus was removed and replaced with a white circle for 200 milliseconds; following this, the next successive colored circle in the sequence was presented. The overall aim of this task is to detect whether participants would learn the repeating sequence. A total of

eight blocks were presented. Within each block there were nine runs of an eight-step sequence. Thus, one block required 72 key-presses. For blocks 1–6 and block 8, the repeating eight-step sequence was presented (i.e. red, green, red, blue, blue, green, yellow and yellow). For block 7, each sequence was random. The software recorded the response latency corresponding to each key-press across the eight blocks. We exported both the median response time and the total number of errors at each block for further statistical analysis.

Executive function

The following tests of executive function were selected on the basis of previously demonstrated sensitivity to profiles within an adult PM-carrier cohort: Hayling sentence completion test, letter-number sequencing (LNS) and excluded letter (EL) verbal fluency. These tasks were explained in previous publications (Cornish *et al.* 2011; Kraan *et al.* 2013c).

Visuospatial function

The tasks selected to measure visuospatial function were selected on the basis of previous demonstrated vulnerabilities in PM-carrier cohorts to tasks that tap into both dorsal stream visuospatial functions and visuospatial working memory (Hocking *et al.* 2012; Keri & Benedek 2009): clock test, mental rotation task and the block design subtest from the WASI-IV.

We employed a written adaptation of the auditory 'visuospatial decision test' (otherwise known as the 'clock test') previously employed in dual-task investigations (Haggard *et al.* 2000; Plummer-D'amato *et al.* 2008). Participants were provided a list of 60 digital times (e.g. 2.45 pm and 1.34 pm) and told to imagine the analog time (i.e. an analog clock face). From the imagined analog image, participants circled one of two answers on a record sheet ascribing the two clock hands as (1) on the same side or (2) on different sides of the clock face. With no access to drawing and reference materials, participants relied solely on the activation of mental imagery to complete this task. Four practice items were completed prior to commencement and the outcome variable was the number of correctly answered items within 60 seconds.

The mental rotation test (Cooper & Shepard 1973) employed for this study was designed to test the participant's ability to detect whether a rotated letter (letter 'R') was in its normal form or a mirror image. The task was administered as a flip book in which two letters (both 'R') were presented side by side. The left-side 'R' was always in its proper form, whereas the right-side 'R' was always rotated and sometimes also a mirror image. Participants were timed and a higher value of correctly answered items indicated better performance. Because of an unexpected ceiling effect in performance, we took total time as the variable for further analyses.

Block design is a subtest from the WASI that measures visuospatial skills. To complete the task, participants are required to reconstruct a series of increasingly complicated visual designs with colored blocks. This task was timed and a higher score indicated better performance. We interpreted the block design WASI *T* score for this investigation.

Psychological symptoms

The following self-report questionnaires were administered on the basis of their sensitivity to psychological symptoms previously reported in female PM-carriers: the self-report Liebowitz Social Anxiety Scale (SR-LSAS), Depression Anxiety Stress Scales (DASS) and Brown Attention-Deficit Disorder (Brown ADD) Scales. These tasks were explained in our previous publication (Kraan *et al.* 2013c).

Procedure

All participants completed the battery of tests in a single session. Neuropsychological tests of executive function and visuospatial ability were administered in random sequence, and symbolic SRTT was administered first. To ensure implicit learning, participants were not provided with training on the symbolic SRTT task. A card showing the key-color associations was placed next to the computer and the participants were told that although they may use this as a guide, the goal was to learn these associations and respond as fast as possible. This task was performed continuously with no breaks in between blocks, and participants were informed prior to the task that it would entail approximately 10 min of attentive key-pressing. Prior to performance there was no mention of any repeating sequence. However, immediately following completion of the task, participants were asked a series of structured questions about their explicit knowledge of the repeating sequence. Specifically, participants were asked the following questions: (1) Did you notice anything different/unusual? (2) Did you notice any patterns or repetitions? (3) Did you notice a repeating sequence? (4) Can you tell me what the repeating sequence was?

Statistical analysis

Data were analyzed using IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA). Normality was checked with the Shapiro–Wilk test. We first conducted independent *t*-test to compare raw scores for differences between PM-carriers and controls on neuropsychological measures and self-reported psychological symptoms (i.e. affect). To analyze awareness of the repeating sequence on the symbolic SRTT, we employed χ^2 analysis to explore group differences in the percentage of individuals noting awareness of the sequence, and for those successfully able to recall >3/4 of the repeating sequence. Mixed model analysis of variance (ANOVA) was conducted to ascertain group effects on symbolic SRTT response latency, with further *t*-tests to explore differences in errors, and time at each block. To index implicit learning we also computed slope (rise over run from B2 to B6), and consistent with previous examinations of performance on the SRTT (Bussy *et al.* 2011), we compared response latency on the random block (B7) with its preceding (B6) and successive (B8) sequenced blocks. This enabled three scores in which a higher score indicated good implicit learning and a preservation of the previously learned sequence following the random block interruption: slope, B7 minus B6 and B7 minus B8. Finally, we analyzed the strengths of correlational associations between SRTT completion time and implicit learning scores with performance on tests of executive function (Hayling's, LNS and EL verbal fluency), visuospatial processing (visuospatial clock test, mental rotation and block design test) and self-reported psychological symptoms (LSAS, DASS and Brown ADD scale). Relationships to age and CGG-repeat length were also examined. To reduce multiple correlations between neurobehavioral measures and each block of the SRTT we took total log-transformed SRTT performance (corrected for slight positive skew in PM-carrier cohort) as a maker of sequence learning. This decision was based on very strong and significant correlations between each block and total SRTT time. Importantly, these correlations were conducted controlling for simple motor reaction time. This task is part of the physiological profile assessment (PPA) and is a measure of the time taken to click on a computer mouse in response to an illuminated red light (Kraan *et al.* 2013b). Because of the large number of comparisons, a relatively stringent Bonferroni correction of $P < 0.01$ was selected for all between group and correlational analyses.

Results

Neuropsychological performance and self-reported psychological symptoms

The analyses revealed significant performance decrements in PM-carriers compared with controls in areas of response inhibition [$t(65) = 3.656$, $P = 0.001$] and EL verbal fluency [$t(65) = -3.482$, $P = 0.001$], and there was a trend toward a PM-carrier deficit in the areas of block design [$t(65) = -2.172$, $P = 0.034$]. Compared with controls, PM-carriers demonstrated elevated symptoms of attention-deficit hyperactivity disorder-predominantly inattentive (ADHD-PI) [$t(65) = 2.828$, $P = 0.007$] and increased symptoms of anxiety [$t(65) = 2.202$, $P = 0.032$] (Table 2).

Table 2: Means and standard deviations for scores on neuropsychological assessments and self-report psychological scales for *FMR1* PM-carriers and control participants

	FMR1 PM-carrier ($N = 34$) M ± SD (range)	Control ($N = 33$) M ± SD (range)	*P*-value
Executive function			
Response inhibition (Hayling's)	9.80 ± 9.06 (0–29)	3.49 ± 4.31 (0–16)	0.001**
Working memory (LNS)	12.65 ± 3.52 (5–20)	13.88 ± 3.22 (9–21)	0.140
EL verbal fluency	15.41 ± 5.36 (3–26)	19.97 ± 5.35 (9–32)	0.001**
Visuospatial function			
Visuospatial decision	20.09 ± 8.88 (5–44)	22.33 ± 6.89 (8–40)	0.253
Mental rotation (milliseconds)	1049.70 ± 384.84 (460–2130)	905.80 ± 405.06 (430–2040)	0.154
WASI block design	55.59 ± 7.11 (42–73)	59.45 ± 7.46 (40–72)	0.034$^{\alpha}$
Psychological symptoms			
Anxiety (LSAS)	45.94 ± 28.86 (6–106)	33.03 ± 18.07 (4–74)	0.032$^{\alpha}$
Depression (DASS_D)	3.91 ± 5.77 (0–21)	2.03 ± 2.27 (0–8)	0.085
ADHD-PI (Brown ADD)	38.32 ± 28.21 (1–101)	22.48 ± 16.21 (0–66)	0.007*

Higher scores for Hayling's errors, mental rotation, LSAS, DASS_D and ADHD-PI reflect an increase in symptoms and worse performance. Alternatively, higher scores on the LNS, EL verbal fluency, visuospatial decision and block design suggest better performance.
ADHD-PI, ADHD-predominantly inattentive from the Brown ADD scale; DASS_D, depression subscale from the DASS; Hayling's, Hayling A + B error score; SD, standard deviation.
$^{**}P < 0.001$, $^{*}P < 0.01$, $^{\alpha}P < 0.05$.

Table 3: Recognition and successful recall of repeating pattern for female PM-carrier and control participants

	PM-carrier ($N = 34$) *N* (%)	Control ($N = 33$) *N* (%)	φ Coefficient	χ^2	*P*-value
Noticed pattern	18 (52.9%)	31 (93.9%)	−0.462	14.326	<0.001**
Recalled >3/4 of pattern	7 (20.6%)	13 (39.4%)	−0.205	2.828	0.093

$^{**}P < 0.001$.

Symbolic SRTT performance

Premutation carriers were significantly less likely than control participants to report that they had noticed the repeating sequence [PM-carrier: 52.9%; control: 93.9%; χ^2 (1, $n = 67$) = 14.326, $P < 0.001$, $\varphi = -0.462$]. When asked to demonstrate this knowledge, there were no significant differences between the proportion of PM-carriers and controls who successfully recalled more than 3/4 of the pattern [PM-carrier: 20.6%; control: 39.4%; χ^2 (1, $n = 67$) = 2.828, $P = 0.093$, $\varphi = -0.205$] (Table 3).

A mixed model ANOVA was conducted to investigate group difference in response time across the eight blocks. This analysis revealed a significant interaction between group and response time (Wilks $\lambda = 0.780$, $F_{7,59} = 2.377$, $P = 0.033$, partial $\eta^2 = 0.220$). Inspection of the graphical output revealed that both groups showed a gradual increase of efficiency up to block 6, and an interruption to this 'learning' at block 7 where the random sequence was introduced. However, while PM-carriers showed little change between B3 and B5 (40.88 milliseconds), control participants demonstrated a steep learning curve during this time period (93.91 milliseconds) and this difference was statistically significant [$t(65) = 2.453$, $P = 0.017$]. Further analyses showed a significant main effect for time (Wilks $\lambda = 0.207$, $F_{7,59} = 32.272$, $P = 0.001$, partial $\eta^2 = 0.793$). The main effect of group was also significant ($F_{1,65} = 13.490$, $P = 0.001$, partial $\eta^2 = 0.172$), with female PM-carriers showing significantly prolonged response latencies across each block relative to controls with normal alleles [B1: $t(65) = 3.596$, $P = 0.001$; B2: $t(65) = 3.474$, $P = 0.001$; B3: $t(65) = 2.937$, $P = 0.005$; B4: $t(65) = 3.211$, $P = 0.002$; B5: $t(65) = 3.831$, $P < 0.001$; B6: $t(65) = 3.013$, $P = 0.004$; B7: $t(65) = 3.344$, $P = 0.002$; B8: $t(65) = 3.573$, $P = 0.001$]. Readers are referred to Table S1, Supporting Information for specific details.

As shown in Fig. 1a, implicit learning of the sequence occurred for both groups, with a reduced reaction time in B8 compared with B7. The impact of the random sequence in B7 was not significantly different between controls and PM-carriers [B7 − B6: PM = 148.44, PC = 182; $t(65) = -0.874$, $P = 0.385$]. Similarly, controls returned to the sequence in B8 with comparable efficiency to PM-carriers [B7 − B8: PM = 52.21, PC = 66.24; $t(65) = -0.609$, $P = 0.545$]. These results suggest that controls and PM-carriers overall retain preservation of the sequence. Analysis of slope produced similar patterns of results with no significant difference between groups on this variable [control: −28.81; PM-carrier: −29.21; $t(65) = -0.060$, 0.952]. Because fast initial learning and good adaptability to the random B7 sequence could reduce slope and implicit learning scores we also examined the percentage of participants with fast, average or slow response latency during block 1. As shown in Fig. 2, fewer

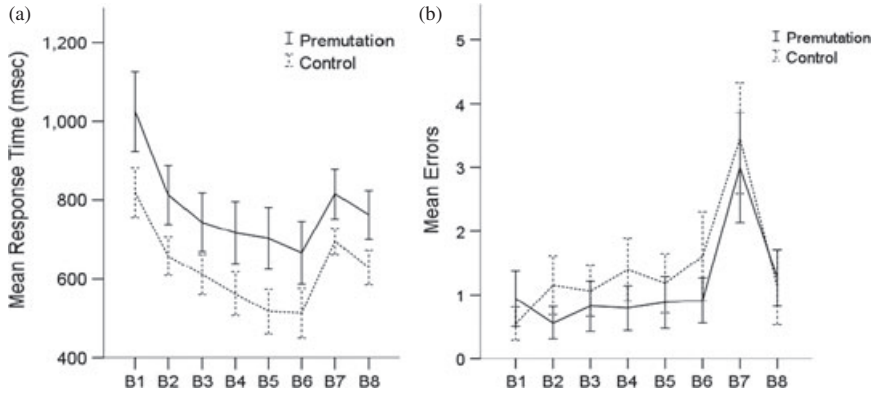

Figure 1: Response latency and errors on the sequence learning task. (a) Mean response time and (b) errors for PM-carrier and control participants across eight task blocks. A repeating sequence was presented from B1 to B6, and again at B8. B7 reflects the introduction of a random sequence. Error bars represent ±2 standard error.

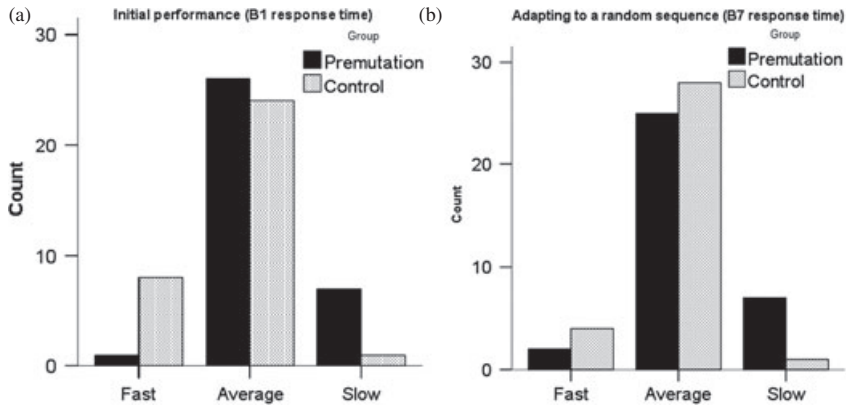

Figure 2: Proportions of slow and fast learners on the sequence learning task. Proportion of PM-carriers and controls as a function of fast, average and slow response latency at (a) the initial block named B1 and (b) the random block termed B7.

PM-carriers than controls responded fast during B1 [χ (1, $n = 67$) = 10.012, $P = 0.005$]. There was also a trend toward a greater percentage of PM-carriers responding slowly on the random block [χ (1, $n = 67$) = 5.323, $P = 0.083$]. As shown in Fig. 1b, there were no significant differences in errors made between PM-carriers and controls (B1: $U = 492$, $P = 0.337$; B2: $U = 407$, $P = 0.037$; B3: $U = 485$, $P = 0.309$; B4: $U = 403$, $P = 0.037$; B5: $U = 498$, $P = 0.399$; B6: $U = 480$, $P = X.283$; B7: $U = 496$, $P = 0.411$; B8: $U = 474$, $P = 0.251$), although there was a trend toward female PM-carriers making less errors than control participants at B2 and B4 (see Table S2 for specific details).

Total symbolic SRTT response latency

Total time for SRTT completion was significantly longer in PM-carriers compared with controls [PM-carrier: 11395; control: 9474.24; $t(65) = 3.889$, $P < 0.001$]. Given that psychiatric rating scales classified some women within a possible clinical range for a mental health disorder – 9 meeting threshold for probable ADHD-PI, 12 probable social phobia and 5 probable depression – we repeated our analyses for SRTT total time after excluding these women (refer to Kraan *et al.* 2013c for methodology). The results retained significance. Further analyses were computed to ensure there were no further confounding variables that may have impacted this result. For example, simple motor reaction time that was significantly slower in female PM-carriers compared with controls [PM-carrier: 0.222; control: 0.220; $t(65) = 2.771$, $P = 0.007$] could have impacted SRTT performance. Importantly, recomputation of this statistic with univariate ANOVA while controlling for reaction time, FSIQ, mood-stabilizing medication use and a probable mental health disorder maintained significance, with PM-carriers exhibiting slower overall performance on the SRTT ($F_{1,65} = 8.765$, $P = 0.004$, partial $\eta^2 = 0.127$). To ensure that this result was not further mediated by attentional profiles as determined by the Brown ADD scale, we rerun this analyses additionally controlling for ADD symptoms. The significant difference remained ($F_{1,65} = 4.954$, $P = 0.030$, partial $\eta^2 = 0.077$).

Correlations to neuropsychological measures and self-reported psychological symptoms

The associations for PM-carriers between performance on the symbolic SRTT and performance on neuropsychological tasks of executive function, visuospatial processing and self-reported psychological symptoms were examined using Pearson's correlational analyses. As shown in Table 4, after controlling for simple motor reaction time, we observed significant correlations between overall symbolic SRTT response latency and worse performance on executive measures (Hayling's: $r = 0.526$, $P = 0.002$; LNS: $r = -0.403$, $P = 0.020$; verbal fluency: $r = -0.308$, $P = 0.081$), deficits on visuospatial measures (visuospatial decision: $r = -0.562$, $P = 0.001$; mental rotation: $r = 0.377$, $P = 0.044$; block design: $r = -0.645$, $P < 0.001$) and higher incidences of

Table 4: Correlational associations between symbolic SRTT response latency and performance on neuropsychological measures of executive function, visuospatial function and affective regulation (controlling for motor reaction time)

Task	PM-carrier Total symbolic SRTT time ($N=34$)	Control Total symbolic SRTT time ($N=33$)
Executive function		
Response inhibition (Hayling's)	0.526*	−0.144
Working memory (LNS)	−0.403$^{\alpha}$	−0.071
EL verbal fluency	−0.308	0.106
Visuospatial		
Visuospatial decision	−0.562**	−0.176
Mental rotation	0.377$^{\alpha}$	−0.038
WASI block design	−0.645**	−0.256
Psychological symptoms		
Anxiety	0.551**	−0.068
Depression	0.584**	−0.130
ADHD-PI	0.456*	0.175

Higher scores for Hayling's errors, mental rotation, LSAS, DASS_D and ADHD-PI reflect an increase in symptoms and worse performance. Alternatively, higher scores on the LNS, EL verbal fluency, visuospatial decision and block design suggest better performance.

$^{**}P < 0.001$, $^{*}P < 0.01$, $^{\alpha}P < 0.05$.

[Correction added on 17 March 2014, after first online publication: $^{***}P < 0.05$ has been changed to $^{\alpha}P < 0.05$].

self-reported psychological symptoms (anxiety: $r=0.551$, $P=0.001$; depression: $r=0.584$, $P<0.001$; ADHD-PI: $r=0.456$, $P=0.008$). For control participants, there were no correlations between symbolic SRTT performance and any of the selected neuropsychological measures or scales. To rule out the possible confound of a probable mental health disorder, we repeated the correlational analyses excluding participants meeting criteria for probable anxiety, depression or ADHD-PI (see Kraan *et al.* 2013c for methodologies). The strength of the correlational values remained significant for associations between symbolic SRTT response latency and performance on block design ($r=-0.590$, $P=0.003$) and visuospatial decision-making tasks ($r=-0.516$, $P=0.012$), suggesting that worse SRTT performance was associated with poorer visuospatial ability irrespective of psychological status. A repeat of this correlational analyses with all individuals ($N=35$) and the implicit learning scores in place of total SRTT response latency showed a positive association between implicit learning and performance on the visuospatial decision task (B7 − B6: $r=0.453$, $P=0.004$), indicating better implicit learning in PM-carriers with good visuospatial ability. In contrast, for controls but not PM-carriers, there were associations between higher implicit learning scores indicative of good learning and better working memory performance (implicit learning B7 − B6: $r=0.371$, $P=0.018$; implicit learning B7 − B8: $r=0.312$, $P=0.041$). Further analyses revealed no correlational associations between any of the selected neuropsychological measures and both age and CGG-repeat length in PM-carriers. However, for controls we did observe a trend association between older age and both reduced SRTT errors ($r=-0.382$, $P=0.028$) and increased total SRTT response latency ($r=0.346$, $P=0.048$), indicating worse performance on the SRTT in older control participants.

Discussion

Here, we show that poorer performance in sequence learning is associated with a range of higher order functions previously shown to be impaired in some females with the expanded *FMR1* allele. Although female PM-carriers appear to have shown preserved implicit learning, the slowed response latency and poor awareness of the repeating sequence suggest a deficit in automaticity required for successful symbolic SRTT performance. Importantly, there were several interrelationships between poorer symbolic sequence learning and a range of cognitive, visuospatial and affective abnormalities in female PM-carriers that differentiated them from controls with normal alleles. The significant associations between working memory and symbolic SRTT implicit learning in controls suggested different strategies of working memory in female PM-carriers. We interpret these data to suggest that cortico-cerebellar involvement may be related to cognitive and affective dysfunctions in some PM-carrier women.

Our results showing significantly poorer response latency on the symbolic SRTT in female PM-carriers compared with controls are consistent with a deficit of automaticity in motor sequencing with a symbolic prime. This finding is in line with previous studies of PM-carriers that have shown poorer performance on the BDS, a multifactorial measure that partially loads onto motor sequencing (Grigsby *et al.* 2008). This is consistent with a previous study that reported that FMRP levels significantly associated with performance on the motor sequencing test from the BDS in female PM-carriers (Loesch *et al.* 2003). Our data do not support an overall deficit in implicit, or unconscious, learning as reflected in the slope and effects from the random block to response latency. However, one possibility is that better performance and more efficient adaptability to B7 in the controls could have contributed to a reduction of slope and implicit learning scores. In line with this, our data from Fig. 2 showed that controls were also more efficient in responding to the random sequence at block 7. This may have inadvertently reduced the implicit learning scores for control participants performing well at the outset, which could have affected our between-group comparisons. Indeed, poor sequence learning performance by PM-carriers was supported by the fact that only 52.9% of PM-carriers were consciously aware of the repeating sequence when compared with 93.9% of controls. Furthermore, the associations between symbolic SRTT response latency and cognitive and affective dysfunctions in PM-carriers, after controlling for motor speed, suggest a unique cognitive contribution to symbolic SRTT performance. It has been suggested that the role of the cerebellum in SRTT performance is in setting up the associations between stimulus and response commands necessary for the development of automatic responding to a symbolic prime (Bo *et al.* 2011a,b). This occurs in

the earliest stages of performance, with the later stages characterized by a disengagement from cerebellar regions and the emergence of cortical and fronto-striatal involvement (Eliassen *et al.* 2001; Honda *et al.* 1998). Indeed, successful SRTT performance typically leads to explicit awareness over time and the recruitment of working memory systems that may enhance performance through strategy (i.e. enumerating colors of sequence). This notion is supported by our present data showing correlations between working memory and implicit SRTT learning in controls. Given the lack of such associations between SRTT implicit scores and working memory in PM-carriers, it is possible that their slowed performance is due to cortico-cerebellar disruption underlying implicit sequencing performance.

Another important finding of the study was that visuospatial functioning correlated with symbolic SRTT performance even after controlling for self-reported psychological symptoms (i.e. anxiety, depression and ADHD-PI). Our previous investigation with female PM-carriers showed that poor executive function was associated with self-reported symptoms of anxiety, depression and ADHD, suggestive of a range of comorbid symptoms associated with core inhibitory control impairments (Kraan *et al.* 2013c). The significant interrelationships between performance on SRTT and visuospatial functioning in female PM-carriers are suggestive of dysfunction in fronto-parietal dorsal stream networks resembling that seen in the FXS full mutation (Kogan *et al.* 2004a,2004b). Indeed, studies with female PM-carriers have shown preserved performance on tasks of ventral stream visuoperceptual function, alongside age- and CGG-dependent deficits on tasks known to tap dorsal stream visuospatial functions (Goodrich-Hunsaker *et al.* 2011a; Keri & Benedek 2009). It is possible that the detected correlations between symbolic SRTT task and visuospatial performance reflect disrupted connectivity in networks important for the visual guidance of movement, such as the closed loops reciprocally connecting dorsal steam networks with the cerebellum (Glickstein 2000).

These findings are consistent with the contention that cerebellar dysfunction may underlie a range of deficits in executive and visuospatial functioning, and psychological symptoms in female PM-carriers (Kraan *et al.* 2013a). This interpretation is consistent with functionally distinct cerebro-cerebellar networks reciprocally connecting cerebellum with the striatum (Bostan *et al.* 2013), limbic system (Schmahmann 2000; Snider & Maiti 1976) and association areas within the prefrontal and parietal cortices (Krienen & Buckner 2009; Middleton & Strick 2001; Ramnani 2006; Strick *et al.* 2009). Moreover, investigations with clinical populations and functional neuroimaging studies have consistently demonstrated that posterior cerebellum (lobule VI and VII) is important for a range of executive, visuospatial, linguistic and affective abilities (Schmahmann 2004; Stoodley *et al.* 2010). The CCAS has been described as dysfunction of posterior cerebellum that results in cognitive, visuospatial and affective deficits, which resemble in a milder form that seen in female PM-carriers (Bernard *et al.* 2012; Stoodley & Schmahmann 2009; Stoodley *et al.* 2010, 2012; Tedesco *et al.* 2011). It has been suggested that feed-forward models, such as those that enable smooth and accurate movement control (Wolpert *et al.* 1995, 1998), are also important for rapid unconscious information processing and automaticity in performance (Koziol *et al.* 2010). Because the posterior cerebellum has strong projections to prefrontal and parietal cortices, disruption to posterior cerebellum has been proposed to result in disorganization, or dysmetria, of cognition (Schmahmann 2004). Overlap between symptoms of CCAS and female PM-carrier phenotypes suggests that tasks with sensitivity to cognitive cerebellar operations may offer clinical utility in identifying women at risk of *FMR1*-associated deleterious phenotypic impact.

The current findings do not support the hypothesis of age or CGG-repeat length effects on symbolic sequence learning in female PM-carriers. One interpretation is that molecular variables known to be associated with increased CGG length such as elevated *FMR1* mRNA, and slight reductions in the percentage of histochemically stained FMRP-positive cells, are not interrelated in sequence learning and cognitive phenotypes in female PM-carriers (Kenneson *et al.* 2001; Tassone *et al.* 2000); however, this claim is tentative given that we were unable to obtain an extensive molecular profile. One explanation is that other molecular and environmental factors may be involved in the manifestation of cognitive and affective phenotypes, for example, psychosocial stressors, carer burden, hormones and X-chromosome inactivation (Abbeduto *et al.* 2004; Hunter *et al.* 2010, 2012; McCarthy *et al.* 2006). The lack of any association between sequencing performance and age in female PM-carriers is suggestive of developmental mechanisms. The posterior region of the cerebellum important for symbolic sequencing is phylogenetically recent, and alongside the prefrontal cortex, developmentally vulnerable with relatively late maturation (Altman & Bayer 1985; Ciesielski *et al.* 1997). Longitudinal neurobehavioral and imaging studies will be important for ascertaining the extent to which impairments in sequencing ability in female PM-carriers are reflective of risk for a neurodegenerative disorder, or subtle and developmentally based phenotypes possibly associated with posterior cerebellum and cortico-cerebellar tracts.

Some limitations require acknowledgment. First, because of the cross-sectional nature of this study, the extent to which problems in sequence learning reflect a stable or age-attenuated phenotype will need to be addressed in prospective longitudinal studies in which both FMRP and *FMR1* mRNA levels are routinely collected. Second, the neural basis of the associations between sequencing and cognitive–affective phenotypes will need to be confirmed in future imaging investigations. They may include voxel-based morphometry and diffusion tensor imaging, and fMRI activation patterns during symbolic SRTT performance and completion of paradigms sensitive to the observed cognitive and affective phenotypes. For example, researchers could use these neuroimaging techniques to examine structural and functional associations between dorsal stream and cerebellar networks in individuals with the expanded *FMR1* allele (Keri & Benedek 2009).

In conclusion, this is the first study to use experimentally driven measures of sequence learning to explore cerebellar involvement in cognitive and affective phenotypes previously reported in female PM-carriers. Our data show a clear slowing

of symbolic SRTT performance, which is reflective of a deficit in automaticity. We conclude that the cerebellum may play a critical role in a range of impairments in response inhibition, visuospatial function and attentional and affective regulation in female PM-carriers of the *FMR1* gene. These data highlight the importance of identifying sensitive measures that may be useful indicators of at-risk profiles in females with the expanded *FMR1* allele.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1: Means and standard deviations for response time on the serial reaction time task for PM-carrier and control participants.

Table S2: Means and standard deviations for errors on the serial reaction time task for PM-carrier and control participants.