Abnormal GABA-mediated and cerebellar inhibition in women with the fragile X premutation

Virginia Conde, Francisco J. Palomar, María José Lama, Raquel Martínez, Fátima Carrillo, Elizabeth Pintado, and Pablo Mir

1 Unidad de Trastornos del Movimiento, Servicio de Neurología y Neurofisiología Clínica, Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocio/Consejo Superior de Investigaciones Científicas/Universidad de Sevilla, Seville, Spain; 2 Departamento de Bioquímica Médica y Biología Molecular, Hospital Universitario Virgen Macarena, Universidad de Sevilla, Seville, Spain; and 3 Centro de Investigación Biomédica en Red Sobre Enfermedades Neurodegenerativas, Seville, Spain

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The fragile X syndrome (FXS) is caused by a repetition over 200 times of the CGG trinucleotide situated in the 5′-untranslated region of the fragile X mental retardation 1 gene (FMR1). The interval between 55 and 199 CGG repeats, which is over the normal range but below full mutation, is named fragile X premutation. Recent studies have focused on the asymptomatic state of fragile X premutation carriers and their potentially relevant preclinical features. However, the underlying neurological mechanisms leading to altered functions in fragile X premutation carriers are still poorly understood. In this study, we wanted to test the hypothesis that asymptomatic women who carry the fragile X premutation present GABAergic and cerebellar abnormalities compared with healthy women without the premutation. We performed noninvasive brain stimulation protocols on both asymptomatic fragile X premutation carriers and controls comprising of measures of GABA<sub>A</sub>- and GABA<sub>B</sub>-mediated intracortical inhibition, afferent inhibition, and cerebellomotor functional interactions. Premutation carriers presented an absence of cerebellar inhibition over primary motor cortex as well as a reduced GABA<sub>B</sub>-mediated intracortical and afferent inhibition compared with healthy nonpremutation controls. These alterations are most probably dependent on a dysfunctional GABAergic mechanism associated with the fragile X premutation condition as previously found in CGG-repeat animal models. Furthermore, the lack of cerebellomotor inhibition could be related to the cerebellar structural abnormalities previously found in carriers of the premutation.

fragile X premutation; transcranial magnetic stimulation; cerebellar inhibition; intracortical inhibition; GABAergic system

THE FRAGILE X SYNDROME (FXS) is caused by a repetition over 200 times of the CGG trinucleotide situated in the 5′-untranslated region of the fragile X mental retardation 1 gene (FMR1; McLennan et al. 2011). This condition, called full mutation, leads to a transcriptional silencing of the gene and a consequent lack of FMR1 protein (FMRP; Hessl et al. 2005). Alleles oscillate between 6 and 55 repeats in the general population, leading to a normal amount of the FMR1 protein (FMRP). In between those ranges of repetitions, there is an interval from 55 to 200 repeats that has been termed fragile X premutation (FXP; Hessl et al. 2005), and it can appear with an estimated frequency between 1:116 and 1:272 in women and between 1:251 and 1:813 in men in the Spanish population (Rodriguez-Revenga et al. 2007; Tassone and Hagerman 2012).

There are abnormal phenotypes associated with the FXP, as fragile X associated tremor/ataxia syndrome (FXTAS) and primary ovarian insufficiency [POI; for review see, Tassone and Hagerman (2012)] and Persani et al. (2009)]. FXTAS is more common in male premutation carriers over the age of 50, and, as a result, both women and men under this age do not usually present any motor or visible cognitive deficit.

Apart from the abnormal phenotypes associated with the premutation condition, recent studies have focused on the asymptomatic state of FXP carriers and their potentially relevant preclinical features such as brain structural correlates or neuropsychological and behavioral abnormalities (Adams et al. 2010; Berry-Kravis et al. 2007; Goodrich-Hunsaker et al. 2011a,b; Hashimoto et al. 2011a,b; Hessl et al. 2005; Hunter et al. 2008a,b, 2009; Johnston et al. 2001; Roberts et al. 2009; Rodriguez-Revenga et al. 2008). In a recent study by Hashimoto et al. (2011b), the authors found a significant grey matter reduction in the cerebellum of asymptomatic male FXP carriers compared with healthy controls without the premutation. Some recent studies have tried to find brain structural correlates of proposed neuropsychological FXP phenotypes in carriers with and without FXTAS (Adams et al. 2010; Hashimoto et al. 2011a). However, the underlying brain function of asymptomatic FXP carriers in a preclinical state is still poorly understood.

Finally, animal models of the FMR1 gene premutation have shed light over the potential neurophysiological correlates of an expanded CGG trinucleotide (Hunsaker et al. 2009; Keri and Benedek 2009, 2010; Kogan et al. 2004), indicating a cerebellar overexpression of GABA<sub>A</sub> receptor subunits as well as of proteins involved in the GABA metabolism as a possible candidate (D’Hulst et al. 2009; Fernandez et al. 2012).

The aim of this study was to examine the brain function of asymptomatic female FXP carriers compared with women without the premutation. Based on the aforementioned research pointing to the cerebellum as well as to GABAergic mechanisms as important factors in FXP, we applied diverse noninvasive brain stimulation protocols to explore GABA<sub>A</sub>- and GABA<sub>B</sub>-mediated inhibitory intracortical circuits within the
primary motor cortex (M1) as well as cerebellomotor functional inhibitory interaction. Our hypothesis was that asymptomatic FXP carriers show altered GABA-mediated as well as cerebellar-motor inhibition (CBI) within M1 in agreement with prior human and animal data.

MATERIALS AND METHODS

Experimental Design

The study comprised the genetic examination of both FXP carriers and control subjects [CGG repetition length, messenger ribonucleic acid (FMR1 mRNA) levels, and activation ratio (AR), for a detailed description of methods see Supplemental Data; Supplemental Material for this article is available online at the J Neurophysiol website] as well as the application of different transcranial magnetic stimulation (TMS) protocols: short-interval intracortical inhibition and facilitation (SICI/ICF, with SICI being GABA-mediated; Ziemann et al. 1996a), measurement of cortical silent period (CSP; presumably mediated by GABA receptors; McDonnell et al. 2006; Werhahn et al. 1999), long-interval intracortical inhibition (LICI; GABA-mediated; McDonnell et al. 2006), CBI as a measure of cerebello-motor functional interaction, and short-latency afferent inhibition (SAI; GABA<sub>A</sub>-mediated, modulated by ACh; Di Lazzaro et al. 2000b, 2007).

Subjects

The study included 13 female FXP carriers (41 ± 6.90 yr; means ± SE) and 13 female controls with similar age (39 ± 6.60 yr; means ± SE). The FXP carrier group was recruited from the Department of Bioquímica Médica y Biología Molecular, Hospital Universitario Virgen Macarena in Seville, Spain.

Nine of the subjects of the FXP carrier group had FXS affected children, while none of the subjects from the control group were caregivers of children with a developmental disorder. FXP carriers were genetically examined to determine the existence of the FMR1 premutation because of being mothers of a FXS-affected child or having relatives with the premutation. Control subjects were studied as well to control for the statistical chance of presenting the premutation allele in general population. All subjects underwent a comprehensive neurological examination before the commencement of the study. Since we were interested in the brain function of asymptomatic FXP carriers, the presence of motor symptoms during the neurological examination was considered as an exclusion criterion.

At the time of the study, none of the subjects were using drugs that could affect motor cortex excitability, emotion, or cognition. Blood tests were carried out to explore thyroid-stimulating hormone, thyroid function, and other clinical aspects, which are believed to be related to the premutation condition (Coffey et al. 2008). Finally, a clinical neurophysiologist performed nerve conduction studies to discard polyneuropathy.

Control subjects were recruited from the general population and hospital workers from the Hospital Universitario Virgen del Rocío in Seville, Spain, where the neurophysiological study was conducted. The genetic study was carried out by the Department of Bioquímica Médica y Biología Molecular, Hospital Universitario Virgen Macarena in Seville, Spain.

Subjects were asked to sign an informed written consent before joining the study, and the experiments were approved by the local ethics committee and were in accordance with the Declaration of Helsinki.

The demographical data of the subjects are summarized in Table 1.

Neurophysiological Study

We used noninvasive brain TMS protocols to examine the motor cortex function and the cerebello-thalamo-cortical functional interaction in FXP carriers compared with healthy subjects. Two Magstim 200 stimulators interconnected by a BiStim module (Magstim; Whitland, Dyfed, UK) were used for double pulse protocols. A single Magstim 200 stimulator and an electrical stimulator (Digitimer DS7, Digitimer; Welwyn Garden City, Hertfordshire, UK) were used for afferent stimulation. A flat figure-of-eight coil with 70-mm external diameter was used for each protocol. Electromyographic (EMG) responses were recorded with Ag-AgCl surface electrodes positioned in a tendon-belly configuration and stored on a personal computer using filters ranging from 2 Hz to 2 kHz (Digitimer D360, Digitimer) and an analog-digital interface (CED 1401 interface; Cambridge Electronic Design, Cambridge, UK) to digitize them for offline analysis with specialized software (Signal software, Cambridge Electronic Design; and NuCursor software, J. Rothwell, Institute of Neurology, University College of London, UK). Trials with background EMG activity were discarded online and repeated. Subjects were placed in a comfortable chair that was height adjusted to allow each subject to place their feet in the most relaxed way. Subjects were asked to maintain their eyes open during the stimulation procedures to reduce the possibilities of falling asleep as well as to minimize fluctuations of motor cortical excitability. A pillow was placed over the lap during the experiments to ensure total relaxation of both hands. TMS protocols were applied following the safety guidelines approved by consensus (Rossi et al. 2009).

We applied TMS over first dorsal interosseous (FDI) and abductor pollicis brevis (APB) muscle representations in the left hemisphere. Right FDI EMG was recorded for SICI, CSP, LICI, and CBI, while FDI and APB EMG coregistration of the right hand was used for SAI in which the APB hotspot was selected.

To find the best FDI and APB, we placed the coil at ∼45° from midline tangentially to the scalp with the handle pointing backwards (Di Lazzaro et al. 2004), adjusting the angle individually if necessary. We checked the elicited motor-evoked potentials (MEPs) according to hand movements, choosing the area where MEPs were of higher amplitude at a lesser intensity and the movement was more FDI- or APB-like depending on the protocol performed. We marked the hotspot on the scalp with a soft-tip pen to have a permanent reference of the target point during the experiment.

We first measured the resting motor threshold (RMT), which was defined as the minimum stimulator output intensity that could elicit a MEP of around 50 μV in 5 out of 10 trials (Rossini et al. 1999). Afterwards, the active motor threshold (AMT) was measured and defined as the minimum stimulator output intensity that could elicit a MEP of ∼200 μV during a voluntary sustained contraction in 5 out of 10 trials. We set the test stimulus (TS) at an intensity in which MEPS were ∼1 mV peak-to-peak amplitude (this intensity was ∼120% of the RMT). Each protocol consisted of 20 TS alone and sets of 10 conditioning stimuli (CS) before TS per interstimulus interval (ISI) in

<table>
<thead>
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<th>Subject</th>
<th>Age</th>
<th>N Affected Children</th>
<th>POI</th>
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</tr>
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<td>0</td>
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</tr>
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</tr>
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</tr>
<tr>
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<td>35</td>
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</tr>
<tr>
<td>6</td>
<td>43</td>
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</tr>
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<td>1</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>13</td>
<td>48</td>
<td>1</td>
<td>No</td>
</tr>
</tbody>
</table>

Age, number of fragile X syndrome (FXS) affected children (N affected children) and primary ovarian insufficiency (POI) of FXP carriers.
a randomized fashion. The order of the applied protocols was counterbalanced across participants.

**SICI.** SICI was carried out according to the double-pulse paradigm first described by Kujirai et al. (1993) with a subthreshold conditioning magnetic stimulus (CS) set at 80% of AMT intensity. ISI of 2 ms and ISI 3 ms were chosen for SICI because GABA\(_A\)-mediated intracortical inhibition is supposed to be maximal at ISIs of 2 to 5 ms, while SICF at motor threshold intensities is usually found at ISIs ranging from 2.5 to 3 ms (Tokimura et al. 1996).

**CSP.** CSP (Calancie et al. 1987) was measured applying the TS over FDI motor hotspot while the subject was performing a voluntary sustained contraction of FDI at \(\approx 30\%\) of maximum contraction. To ensure the maintenance of the contraction, we asked the subjects to squeeze a 2-cm block between the thumb and the index finger to contract mostly FDI. We collected 15 responses measured from the onset of EMG suppression until the first visible reoccurrence of voluntary motor activity.

**LICI.** LICI was measured applying a suprathreshold CS set at 110% of RMT intensity over FDI motor hotspot 100, 150, and 250 ms before a TS (Valls-Sole et al. 1992). Intracortical inhibition has been reported to occur at ISIs from 50 to 200 ms.

**CBI.** We followed a modified protocol of the CBI paradigm first described by Ugawa et al. (1995). A CS was applied with a second flat figure-of-eight coil with a 70-mm external diameter over the right hemisphere hotspot determined at 3 cm right and 1 cm below to the inion (Koch et al. 2008). ISIs of 3, 5, 7, and 9 ms before a TS applied on the left hemisphere hotspot for FDI were chosen (a significant inhibition with respect to the TS has been shown between the intervals of 5 and 7 ms). Since in this protocol it is necessary to use two coils at the same time, the BiStim module was removed and the TS was adjusted to obtain a MEP of \(-1\) mV peak-to-peak. The RMT for the right FDI hotspot was measured and the CS was then set at 90% of the right RMT.

**SAI.** An electrical stimulator was placed over the right median nerve with a standard stimulation block at the wrist (anode distal), and the electric stimulus (ES) intensity was adjusted to elicit a 1 mV peak-to-peak EMG response on the right APB (\(\approx 3\) times the perceptual threshold) (Quartarone et al. 2009). The duration of the ES was 200 \(\mu\)s. An ES was applied at 18, 20, 22, and 30 ms before a magnetic suprathreshold CS applied over the left APB hotspot. We expected an inhibition of the TS at \(\approx 20\) ms ISI according to Tokimura et al. (2000).

**Statistical Analysis**

We divided the data into two groups: healthy subjects (control) and FXP carriers. Shapiro-Wilk test was used to assess assumption of normality of the obtained data before the use of parametric tests. TMS intensities and clinical and demographic data were analyzed using \(\chi^2\)-squared and Student’s \(t\)-tests for independent samples \(t\)-test for independent measures depending on data type. All ISI results of SICI, LICI, CBI, and SAI were analyzed using normalized-to-test data while results of CSP were analyzed in raw (ms) data. The result of CSP was evaluated using a \(t\)-Student comparison between the two different groups of study. Results of all other TMS protocols were compared using two-way repeated measures ANOVA with factor group (FXP carriers vs. control) as a between-subject factor. For SICI, LICI, CBI, and SAI protocols, we used ISI as a within-subject factor. Mauchley’s test assessed sphericity and Greenhouse-Geisser correction was used for nonspherical data. Significant main effects in ANOVA were followed by post hoc pairwise comparisons (independent-samples \(t\)-test analysis) with Bonferroni correction for multiple comparisons (adjusted Bonferroni corrected p-values are presented). Additional independent samples \(t\)-tests were used to compare baseline 1-mV MEPs data between groups. Finally, Pearson’s (normal distribution) or Spearman’s (nonnull distribution) correlations were performed to account for potential correlations between demographical (age, children affected with FXS) and clinical (presence of POI) data, genetic profile (number of CGG repetitions, AR and \(FMR1\) mRNA levels) and neurophysiological results (SICI, LICI, CSP, SAI, and CBI).

## RESULTS

Participants from both groups were not significantly different in age (independent samples \(t\)-test, \(P > 0.05\)).

### Clinical Examination

None of the FXP carriers presented signs of polynephropathy or a clinical history of thyroid dysfunctions; the thyroid-stimulating hormone levels of all FXP carriers were within normal ranges (0.4–4.00 \(\mu\)U/ml) and 4 out of 13 carriers presented POI (see Table 1).

### Genetic Study

Our cohort of female premutation carriers showed a range of allele sizes from 70 to 170 repeats. In accordance with Tassone et al. (2000), alleles with range of 55–99 repeats are designated as “low premutation” and alleles with a range of 100–200 repeats as “high premutation.” In this study, eight women had low premutation alleles and five women high premutation. The relative \(FMR1\) mRNA level for each individual compared with controls is shown in Table 2. Increases from 1.8- to 7.5-fold are observed. Considering the small size of the sample we have not made any correlation between CGG number and \(FMR1\) mRNA levels. We have also analyzed the AR measuring the methyl-ation pattern at different loci (see MATERIALS AND METHODS). For a more detailed profile of the premutation carriers, see Table 2. None of the control participants was identified with the premutation condition.

### Neurophysiological Study

All TMS interventions were well tolerated by the subjects without the report of any unexpected discomfort. No significant differences for RMT, intensity to evoke 1-mV MEP (maximum stimulator output), and median nerve stimulation (in SAI) were found between FXP carriers and controls (\(P > 0.05\)). The data were normally distributed in SICI, LICI, CSP, SAI, and CBI.

### Table 2. Genetic results of FXP carrier subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>CGG Triplet Repeats</th>
<th>(FMR1) mRNA</th>
<th>AR, %</th>
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<tbody>
<tr>
<td>1</td>
<td>103</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>79</td>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>79</td>
<td>6.9</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>2.2</td>
<td>45</td>
</tr>
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<td>5</td>
<td>80</td>
<td>3.2</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>12</td>
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</tr>
<tr>
<td>13</td>
<td>95</td>
<td>4.2</td>
<td>50</td>
</tr>
</tbody>
</table>

Summary of genetic results of FXP carrier women in the study. CGG triplet repeats of the premutated alleles are shown. \(FMR1\) mRNA shows levels of \(FMR1\) mRNA. Activation ratio (AR, %) includes the AR of each FXP carrier (as %normal \(FMR1\) allele as active allele). Please mind that due to technical reasons not all measures could be performed in all participants (missing data are represented by a dash).
CBI, and SAI protocols (Shapiro-Wilk test for normality, \( P > 0.05 \) in all cases for both control and FXP carriers groups). Five participants had to be excluded from SAI measures due to lack of APB responses of at least 0.5 mV at 70% of maximum stimulator output or high interstimulus variability (3 control participants and 2 FXP carriers). Two control participants did not undergo CBI testing due to technical reasons. For a visualization of within-group and between-groups data variability see supporting material (scatter plots A–D).

SICI. We found a significant ISI effect in the amplitude of the overall conditioned MEPs compared with unconditioned ones \([F(2,23) = 76.093, P < 0.001]\), a significant group effect \([F(1,24) = 5.587, P = 0.027]\), and an ISI × group interaction \([F(2,23) = 3.596, P < 0.044]\) in the repeated-measures ANOVA analysis, indicating an overall difference between groups in the SICI effect. The intragroup analysis revealed a statistically significant inhibition at 2 ms \((P < 0.001)\) and 3 ms \((P < 0.001)\) in the control group \(%\text{inhibition} 2 \text{ms}: 57 \pm 22\%\) (means ± SD); \%inhibition at 3 ms: 72 \± 21\%\) as well as a statistically significant inhibition at 2 ms \((P < 0.001)\) and 3 ms \((P < 0.001)\) in the FXP carriers group \(%\text{inhibition} 2 \text{ms}: 34 \pm 21\%\); \% of inhibition at 3 ms: 54 \± 38\%). Post hoc \(t\)-test analysis with Bonferroni correction of SICI ISIs showed statistically significant differences between control and FXP carriers groups at 2 ms \((P = 0.003)\) but not at 3 ms \((P = 0.144)\); see Fig. 1A). More specifically, FXP carriers had 17\% less inhibition than control subjects at 2-ms ISI and, although not significantly different, 18\% less inhibition at 3-ms ISI. No significant differences were found in baseline 1-mV MEPs \((P > 0.05)\).

CSP. No significant differences were found in CSP length between groups \([\text{FXP carriers}: 127.34 \pm 5.80\%\); \text{controls}: 122.97 \pm 9.43\%; \text{ANOVA} (1,22) = 0.156, \( P > 0.05)\].

LICI. The repeated-measures ANOVA revealed significant effects for factor ISI only \([F(3,22) = 10.691, P < 0.001]\), while no significant effects could be addressed for factor group \([F(1,24) = 2.335, P = 0.140]\) or ISI × group interaction \([F(3,22) = 1.023, P < 0.402]\). Statistically significant inhibition at 100 ms \((P < 0.001)\) and 150 ms \((P < 0.001)\), but not at 250 ms \((P = 0.270)\), was observed in the control group \(%\text{inhibition} 100 \text{ms}: 60 \pm 41\%\); \%inhibition at 150 ms: 57 \± 33\%). The FXP carriers group, on the other hand, showed statistically significant inhibition at 100 ms \(%\text{inhibition} 2 \text{ms}: 42 \pm 50\%) but not at 150 ms \((P = 0.266)\) and 250 ms \((P = 0.721)\); see Fig. 1B). Because we were unable to find a group effect and/or an ISI × group interaction, post hoc pairwise between-groups comparisons were not performed. The baseline analysis revealed no significant differences between groups regarding 1-mV MEPs \((P > 0.05)\).

CBI. Although we did not find a general significant ISI effect in the repeated measures ANOVA analysis \([F(4,19) = 1.749, P = 0.183]\), a significant group effect \([F(1,22) = 7.452, P = 0.012]\) as well as an ISI × group interaction \([F(4,19) = 4.663, P = 0.009]\) indicated an overall significant difference between groups in the CBI effect. A statistically significant inhibition at 3 ms \((P = 0.009)\) and 5 ms \((P = 0.001)\) was observed in the control group \(%\text{inhibition} 3 \text{ms}: 17 \pm 18\%\); \%inhibition at 5 ms: 29 \± 21\%). No significant inhibition was found at any of the ISI tested in the FXP carriers group indicating that, in contrast to control participants, asymptomatic premutation carriers did not show the expected CBI at any of the tested ISIs.

Post hoc pairwise between-groups comparisons showed statistically significant differences between control and FXP carriers groups at 5 ms \((P < 0.001)\), Bonferroni corrected) only, with control subjects showing 41\% more inhibition than FXP carriers. A nonsignificant trend was found in the condition of 3 ms \((P = 0.044)\); see Fig. 2). No significant differences in baseline 1-mV MEPs were found between groups \((P > 0.05)\).

SAI. A significant ISI effect \([F(4,16) = 22.513, P < 0.001]\) and an overall ISI × group interaction \([F(4,16) = 4.609, P = 0.011]\) was revealed by the repeated-measures ANOVA analysis, but we were unable to find a significant group effect \([F(1,19) = 0.191, P = 0.667]\). Statistically significant inhibition at 20 ms \((P = 0.001)\) was only observed in the control group \(%\text{inhibition}: 54 \± 34\%\), while no statistically significant inhibition at any of the ISIs tested was observed in the FXP carriers group. Post hoc pairwise comparisons with Bonferroni correction showed a significant difference between controls and FXP carriers at 20-ms ISI \((P = 0.039)\); see Fig. 3). There were no differences in baseline 1-mV MEPs \((P > 0.05)\) as well as in the intensities used to stimulate the median nerve.

Fig. 1. A: motor-evoked potential (MEP) amplitude changes in the right first dorsal interosseous (FDI); \% of inhibition 2 ms: 42 \um). Statistically significant short-interval intracortical inhibition (SICI) double-pulse protocol at 2- and 3-ms interstimulus interval (ISI) between pulses. Black bars represent the fragile X premutation (FXP) carriers group and white bars represent the control group. Please note that only the interval of 2-ms ISI resulted in a significant difference between groups (*). Both groups showed significantly reduced inhibition (2- and 3-ms ISI). Bars indicate \% of inhibition 2 ms: 57 \± 22\% as well as a significant inhibition at 3-ms ISI. No significant differences were found between control subjects showing \% of inhibition at 3-ms ISI. No significant differences were found in baseline 1-mV MEPs \((P > 0.05)\).

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and to a higher intersubject variability in the FXP group compared with the control group within the LICI protocol. The reduced intracortical inhibition observed as well as the lack of CBI in FXP carriers could be due to two separate mechanisms or could be codependent. Thus the reduced amount of SICI could theoretically be caused by the lack of CBI and vice versa, since cerebellum and motor cortex are functionally interconnected. However, no significant correlations could be observed between SICI (2 ms) and CBI (90% of RMT, 5 ms) MEP values, indicating that these altered mechanisms are most probably independent of each other. Even although we performed a high number of TMS protocols, it is important to keep in mind that the reported intergroup differences were corrected for multiple comparisons (Bonferroni correction), and thus were considered (specially in the case of CBI, where the $P$ value was $< 0.001$) highly significant and hardly dependent on the number of protocols performed.

**GABAergic System and FMR1 Premutation**

In an animal model of the FXP, D’Hulst et al. (D’Hulst et al. 2009) found an upregulation of several subunits of the GABAA receptor as well as of proteins involved in the GABAergic metabolism within the cerebellum of expanded CGG-repeat mice in a preclinical state. In humans, GABA-mediated mechanisms can be studied with double-pulse TMS protocols. Such protocols involve the application of a conditioning stimulus that activates GABAergic inhibitory interneurons and suppresses cortical output evoked by a subsequent test stimulus (Fitzgerald et al. 2009). Our FXP carriers showed a decreased SICI at 2-ms ISI compared with healthy controls, while no significant differences between groups were found at 3-ms ISI. The lack of between-groups differences at 3-ms ISI could be due to the influence of SICF at both 2- and 3-ms intervals, since SICF has been usually reported between 2.5-and 3-ms ISI (Tokimura et al. 1996). Both FXP carriers and controls showed a stronger inhibition at 3 ms compared with 2-ms ISI (control group % inhibition: 2 ms: 57%, 3 ms: 72%; FXP carriers % inhibition: 2 ms: 34%, 3 ms: 54%), which could indicate a higher influence of SICF at 2 ms in both groups. Moreover, the
standard deviation at 3-ms ISI in FXP carriers was higher than the standard deviation observed at 2-ms ISI in the same group, while controls showed similar response variability at both ISIs. This higher variability could under-power potential differences between groups seen at 3-ms ISI. Nonetheless, both groups showed an inhibitory effect at both 2- and 3-ms ISI while no SICF was observed at these intervals. The rapid inhibition of SICI seems to coincide with the time window of inhibitory post-synaptic potentials produced by the GABA\textsubscript{A} receptors and decreases with the use of GABA\textsubscript{A} antagonists (Di Lazzaro et al. 2000a; McCormick 1992), this being the reason why it has been considered GABA\textsubscript{A}-dependent. Similarly, SAI reflects cholinergic mechanisms as well as their presynaptic GABA\textsubscript{A}-mediated modulation; however, it seems that different subtypes of GABA\textsubscript{A} receptors are involved in SICI and SAI (Di Lazzaro et al. 2007). In our case, both SICI and SAI were significantly different from controls, indicating the existence of a dysfunction of GABA\textsubscript{A}-mediated neuromodulation in this group that might include different GABA\textsubscript{A} receptor subtypes. In the case of LICI, the mechanisms activated by it seem to depend on GABA\textsubscript{A} modulation (McCormick 1989; McDonnell et al. 2006; Werhahn et al. 1999). Even though GABA\textsubscript{B} involvement has been suggested in the full-mutation syndrome, no involvement has yet been found in the premutation state. In our cohort, there seemed to be a lack of GABA\textsubscript{B}-mediated inhibition that, however, did not result in significant differences compared with controls. This failure in reporting significant between-groups differences renders the interpretation of the role of GABA\textsubscript{B} receptor subunits in the FXP difficult. Furthermore, the within-group variability in FXP carriers regarding the amount of GABA\textsubscript{B}-mediated inhibition was quite high and was probably responsible for the lack of significant differences compared with the control group. Finally, there was no difference in CSP length between FXP carriers and controls. The CSP has also been linked to GABA\textsubscript{B} mechanisms (Ziemann et al. 1996b), although its dependence on GABA\textsubscript{B} has not been consistently addressed in the literature (McDonnell et al. 2006) and could, at least partially, also be related to GABA\textsubscript{A} activity (Ziemann et al. 1996a). Taken together, the lack of significant differences between FXP carriers and controls in LICI and CSP could also indicate a more stable GABA\textsubscript{A} neuromodulation in premutation carriers. In agreement with D’Hulst et al. (2009), thus we have found a remarkable dysfunctional inhibitory drive of the cerebellum over M1, while GABA\textsubscript{A}-mediated intracortical inhibition was preserved although significantly reduced compared with controls that were not FXP carriers. Moreover, there was a significant absence of SAI in the FXP carriers group compared with controls, presumably linked to an overall GABA\textsubscript{A}-receptor dysfunction. We would like to point out that, even though SICI, SAI, and CBI alterations have been previously found in a variety of neurological disorders (Brighina et al. 2009; Ni et al. 2010; Pinto et al. 2003) and thus could represent unspecific alterations associated with neurological malfunction, the FXP carriers included in this study did not present any motor or cognitive dysfunction by the time of the measurements.

Interestingly, a negative correlation was found between the amount of inhibition present in SICI at 2-ms ISI and the percentage of AR of normal \textit{FMRI} allele. Since the functional relevance of the CGG repetition length in female carriers of the \textit{FMRI} premutation is closely dependent on how much of the normal allele is activated, this correlation points to the linkage between the amount of the expanded allele that is active and its potential brain functional outcome as a reduced intracortical GABA\textsubscript{A}-mediated inhibition. Finally, even though D’Hulst et al. (2009) explained their findings based on the cerebellar phenotype of patients with FXTAS, the expanded CGG-repeat mice examined in their study did not present any clinical symptoms related to the disease at the time of the experiments. The authors suggested that the GABAergic upregulation in the cerebellum was a potential marker of FXTAS in FXP carriers. However, since the expanded CGG-repeat mice were asymptomatic, the cerebellar abnormality could be associated with the premutation itself in accordance with the results of Hashimoto et al. (Hashimoto et al. 2011b) in male FXP carriers.

\textbf{Role of the Cerebellum in \textit{FMRI} Premutation}

The abnormal CBI observed in the FXP group is in line with previous findings that point at the cerebellum as a potentially relevant structure in both the preclinical and clinical state of FXP carriers. For example, in a recent study by Hashimoto et al. (2011b), the authors examined the grey matter volume of male \textit{FMRI} premutation carriers with and without FXTAS, compared with that of healthy controls. In patients with FXTAS, profound grey matter loss was found both in the cerebellum and in cortical and subcortical areas compared with controls. Furthermore, despite a lack of significant differences in a whole brain comparison between controls and asymptomatic premutation carriers, a region of interest analysis showed a significant grey matter loss in the cerebellum for the premutation group only, which suggests structural cerebellar abnormality in male premutation carriers before the onset of FXTAS. Although only women participated in our study, the results of Hashimoto et al. (2011b) seem to agree with the lack of CBI observed in our subjects and thus support the idea of an abnormal cerebellar function in relation to the \textit{FMRI} premutation that might be present in both male and female premutation carriers.

A limitation of the present study is that the relatively small number of participants reduces the potential to include a neuropsychological examination of the FXP carriers and underpowers neurophysiological measures that show high inter-subject variability. New studies are needed to understand the functional correlates of such abnormal mechanisms found in FXP carriers (neuropsychological profile) as well as to expand the nonsignificant results found in this study. Moreover, we could only have access to women with the fragile X premutation and not men who were premutation carriers. Similar studies with a more heterogeneous sample are needed to elucidate whether neurophysiological abnormalities in premutation carriers are gender dependent. Furthermore, additional experiments targeting different neuromodulators such as ACh or glutamate are needed to elucidate the role of these mechanisms in FXP.

In conclusion, we have shown an altered cerebellar and intracortical inhibition associated with the FXP condition, which is most probably dependent on GABAergic mechanisms that are potentially dysfunctional in expanded CGG-repeat carriers. This, in turn, reinforces the role of the cerebellum and the GABAergic system in human FXP carriers with potential...
implications in future treatment directions in preclinical states, where a specific neurophysiological phenotype could influence the prophylaxis or prognosis of FXF-related disorders. Additionally, it underpins the potential of TMS as a tool for the inspection of preclinical states in asymptomatic carriers of well-known genetic premutations such as the FMR1 premutation. However, more studies are needed to elucidate the interactions between GABAergic and cerebellar function and other genetic and behavioral aspects linked to the FXP condition as well as longitudinal studies that examine premutation carriers from an asymptomatic to a clinical state.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.C., F.P., and P.M. conceived and designed the research; F.P. and P.M. interpreted results of experiments; V.C. prepared figures; V.C. drafted manuscript; E.P. and P.M. approved final version of manuscript; E.P. and P.M. contributed reagents/materials/analysis tools.

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