

A NULL MUTATION FOR *Fmr1* IN FEMALE MICE: EFFECTS ON REGIONAL CEREBRAL METABOLIC RATE FOR GLUCOSE AND RELATIONSHIP TO BEHAVIOR

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Abstract—As a measure of functional activity we determined regional cerebral metabolic rate for glucose ($rCMR_{glc}$) in adult, female wild type and fragile X (*Fmr1* null) mice homozygous and heterozygous for the null mutation. To ascertain if the sexes differ with respect to the severity of the effects of the mutation we compared our results with results of our previous study on male *Fmr1* null mice [Qin M, Kang J, Smith CB (2002) Increased rates of cerebral glucose metabolism in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A* 99:15758–15763.]. In contrast to the male *Fmr1* null mouse, $rCMR_{glc}$ was unchanged in the homozygous female except in the dorsal raphe where $rCMR_{glc}$ was increased by 36%. There were no differences in $rCMR_{glc}$ between heterozygous and wild type female mice. We compared male and female mice for effects of the null mutation on behavior. We found that the female *Fmr1* null mouse is similar to the male with deficits in performance on a passive avoidance task, general hyperactivity, and increased susceptibility to audiogenic seizures. Both homozygous and heterozygous female mice exhibited hyperactivity and increased susceptibility to seizures, whereas only the homozygous mice had a deficit on the passive avoidance test. Male *Fmr1* null mice had a tendency for lower anxiety-like behavior in an open field, whereas this was not evident in females. Compared with male wild type, male *Fmr1* null mice also had a diminished acoustic startle response at higher stimulus intensities, whereas all three female genotypes had responses similar to those of male *Fmr1* null mice. Whether estrogen affords female *Fmr1* null mice some protection from the effects of the mutation remains to be determined. Published by Elsevier Ltd on behalf of IBRO.

Key words: functional activity, $rCMR_{glc}$, open field activity, passive avoidance, audiogenic seizures, acoustic startle.

Fragile X syndrome (FraX) is the most common inherited form of mental retardation in males with an estimated frequency of 1/4000. It is caused by the absence of the fragile X mental retardation protein (FMRP) encoded by

the silenced fragile X mental retardation gene (*FMR1*) on Xq27.3. Expansion of an unstable trinucleotide CGG repeat in the 5'-untranslated region of *FMR1* to more than 200 repeats leads to gene methylation and transcriptional silencing of *FMR1*. Carriers with alleles with 55–200 repeats are referred to as premutation carriers; the normal allele has fewer than 54 repeats. The carrier frequency in the general population is approximately one in 250 females and one in 760 males (Rousseau et al., 1995). FraX phenotype includes cognitive impairments from learning disabilities to severe mental retardation (Rousseau et al., 1994); behavioral dysfunction such as hyperactivity, social anxiety, attention problems and autistic-like behavior (Miller et al., 1999); and subtle physical abnormalities including a long face, prominent ears, prominent forehead and jaw, and in males, macroorchidism (Hagerman, 2002). Nearly all males with the full mutation have severe to mild mental retardation, whereas in females the prevalence of mental retardation is estimated to be 55% (Cronister et al., 1991; Rousseau et al., 1994).

The mouse model of FraX was created by insertion of a nonfunctional *Fmr1* gene in exon 5 (Bakker et al., 1994) to produce a null mutation for *Fmr1*. Although aberrantly spliced transcripts containing *Fmr1* mRNA may be present (Yan et al., 2004) these mice do not have detectable levels of FMRP (Bakker et al., 1994; Peier et al., 2000). In females, only mice homozygous (Hmz) for the mutation are null for *Fmr1*. Heterozygous (Htz) female mice should exhibit mosaicism with respect to the mutation. Mosaicism is the result of X-inactivation, i.e. each cell will express only one X chromosome, the other is inactivated early in development. Because the inactivation is random and occurs after several thousand cells have formed in the embryo, every female is a mosaic of clonal groups of cells expressing one or the other X chromosome. Most studies characterizing the FraX mouse model have used male mice. The mouse model has many of the characteristics of patients with FraX including immature dendritic spines (Comery et al., 1997; Irwin et al., 2000), behavioral deficits (D'Hooge et al., 1997; Fisch et al., 1999; Paradee et al., 1999; Dobkin et al., 2000; Peier et al., 2000; Van Dam et al., 2000; Mineur et al., 2002; Qin et al., 2002), and macroorchidism (Bakker et al., 1994; Qin et al., 2002). To our knowledge female FraX mice have been the subjects of only one earlier study (Musumeci et al., 2000).

Our previous study of the male *Fmr1* null mouse model was directed at elucidating brain regions or networks that have altered functional activity, since functional activity may be an indicator of brain regions involved in behavioral

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Abbreviations: CMR_{glc} , global cerebral metabolic rate for glucose; DG, deoxyglucose; FMRP, fragile X mental retardation protein; *Fmr1*, fragile X mental retardation-1 gene; FraX, fragile X syndrome; Hemz, hemizygous; Hmz, homozygous; Htz, heterozygous; PCR, polymerase chain reaction; $rCMR_{glc}$, regional cerebral metabolic rates for glucose; WT, wild-type.

manifestations of the syndrome. We determined regional cerebral metabolic rates for glucose ($rCMR_{glc}$) as a measure of functional activity (Sokoloff, 1977), and we reported that in adult, male hemizygous (Hemiz) *Fmr1* null mice, $rCMR_{glc}$ were increased over rates in wild-type (WT) littermates. Differences in $rCMR_{glc}$ ranged from 12% to 46%, and the greatest differences occurred in regions of the limbic system and in primary sensory and posterior parietal cortical areas (Qin et al., 2002). The male *Fmr1* null mice exhibited hyperactivity and deficits in the passive avoidance test of learning and memory (Qin et al., 2002). We have expanded our studies to include female *Fmr1* null mice to investigate possible sex differences in the phenotypic expression of the null mutation and the effect of heterozygosity. We report here that the effects of the *Fmr1* null mutation on $rCMR_{glc}$ in females are considerably different than those found in males. We have tried to understand these differences by studying behavioral effects of the mutation in both sexes. We present our results of studies of behavior in an open field, performance on a passive avoidance test, tests of acoustic startle response, and susceptibility to audiogenic seizures in both male and female WT and *Fmr1* null mice.

EXPERIMENTAL PROCEDURES

Animals

All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee. We were careful to minimize both the number of animals used and their suffering. FVB/NJ-*Fmr1*^{tm1Cgr} breeding pairs (Htz females and Hemz males) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Htz female and WT or Hemz male offspring were mated to provide offspring in five experimental groups: Htz females, Htz females, WT females, Hemz males, and WT males. All mice were housed in a central facility and maintained under controlled conditions of normal humidity and temperature with standard alternating 12-h periods of light and darkness. Food (NIH-31 rodent chow) and water were provided *ad libitum*. All mice studied were between 16 and 23 weeks of age.

Genotyping

Genomic DNA was extracted from a section of tail taken from each animal (Puregene, Gentra Systems, Inc, Minneapolis, MN, USA). Primers to screen for the presence or absence of the mutant allele were 5'-ATCTAGTCATGCTATGGATATCAGC-3' and 5'-GTGGGCTCTATGGCTTCTGAGG-3'. The DNA, a polymerase chain reaction (PCR) buffer, and *Taq*DNA polymerase (AmpliTag Gold, Applied Biosystems, Foster City, CA, USA) were combined and subjected to 35 cycles at 95, 62, and 72 °C. After amplification, the products were separated by electrophoresis on a 1.5% agarose gel at 100 V for 1 h. The PCR product at ≈800 bp indicated the presence of the null allele. To screen for the presence or absence of the WT allele, the S1 (5'-GTGGTTAGCTAAGTGAGGATGAT-3') and S2 (5'-CAGGTTTGGGATTAA-CAGATC-3') primers were used with the same PCR buffer. The presence of the PCR product at 465 bp indicated the presence of the WT allele. It was necessary to use both sets of primers to genotype the female animals.

Locomotor activity in an open field

Locomotor activity was evaluated by placing mice in an open field consisting of a clear Plexiglas box (40×40×30 cm) with a black floor in standard room light. Activity was recorded for 30 min, quantified by a computer-operated tracking system of 16 photo-beams per side (TruScan System, Coulbourn Instruments, Allentown, PA, USA), and analyzed at 6-min intervals. Total distance moved, distance moved in the margins of the field (within 6.25 cm of walls), time spent in the center of the field (area >6.25 cm from walls), and number of entrances into the center zone were measured. The center time/total time can be used as an index of anxiety-related responses (Crawley, 1989).

Passive avoidance

Animals were trained in a passive avoidance apparatus (Small Animal Shocker, Coulbourn Instruments) with one lighted and one dark compartment separated by a guillotine door. On training day, each mouse was placed in the lighted compartment and given access to the dark compartment by raising the guillotine door after 5 s. On entrance into the dark compartment, the guillotine door was closed, and an electric foot-shock (0.2 mA for 1 s) administered. The mouse was removed from the apparatus after 5 s and returned to its home cage. Mice that did not enter the dark compartment within 60 s were eliminated from the study. After 24 h, each animal was placed in the lighted compartment and the latency to enter the dark compartment was recorded up to a maximum of 300 s.

Acoustic startle response

Mice were housed individually 24 h before the test. Startle reactivity was measured with the Startle Reflex System (SR-Laboratory, San Diego Instruments, San Diego, CA, USA). The system monitors animal movements within the cylindrical animal enclosure with a 12-bit motion sensor. Each mouse was acclimated for 5 min to the cylindrical enclosure placed inside a soundproof isolation cabinet. Background noise was 60 dB. The test consisted of 10 stimuli at each of seven intensities (0, 70, 80, 90, 100, 110, 120 dB). The order of the stimuli was determined for each animal with a random number generator. The inter-stimulus interval was between 20 and 40 s. At each stimulus intensity 150 readings were taken at 1 ms intervals and the V_{max} (the peak response in the 150 ms) was used as the stimulus response level. For each animal at each stimulus intensity the mean V_{max} of the 10 measurements was used. All tests were conducted between the hours of 12:00 and 16:00. Mice used in the acoustic startle response test were subjected to audiogenic seizure induction one to two weeks later.

Audiogenic seizure induction

Mice were placed in a sound-attenuating chamber equipped with a glass door for observation. After a 5 min habituation period, animals received a 120 dB auditory stimulus (2–20 kHz) for 1 min. The following responses for each animal were noted: no response, wild running, jumping, clonic or tonic seizure, and respiratory arrest. All tests were done between the hours of 17:00 and 20:00, and each mouse was tested only once. Scorers were blind to the genotype.

Surgical preparation of animals

Mice were prepared for metabolic studies by insertion under light halothane anesthesia of polyethylene catheters (PE-10) into one femoral artery and one femoral vein as previously described (Smith and Kang, 2000). Mice recovered from the surgery for 18–20 h during which time they could move freely within the

confines of the cylindrical Plexiglas container seven inches in diameter. Food and water were available *ad libitum*.

Physiological variables

Mean arterial blood pressure, hematocrit, rectal temperature, and arterial plasma glucose concentrations were measured to evaluate each animal's physiological state as previously described (Qin et al., 2002).

Determination of rCMR_{glc}

Cerebral metabolic rate for glucose was determined by the autoradiographic [¹⁴C]deoxyglucose (DG) method as previously described for the mouse (Qin et al., 2002). Mice remained freely moving in the cylindrical Plexiglas container for the study. The experimental period was initiated by an i.v. pulse injection of 120 μCi/kg of 2-deoxy-D-[1-¹⁴C]glucose (specific activity, 50–55 μCi/mmol; PerkinElmer Life Sciences, Inc., Boston, MA, USA) contained in ≈40 μl of physiological saline, and timed arterial samples were collected during the following 45 min for determination of the time courses of the plasma glucose and [¹⁴C]DG concentrations. At the end of the experimental interval, mice were killed by an i.v. injection of a lethal dose of sodium pentobarbital, and brains were removed rapidly and frozen in isopentane (−40 °C). Serial sections, 20 μm thick, were cut in a Leica 1850 cryostat (Leica Microsystems, Inc., Bannockburn, IL, USA) at −18 °C, thaw mounted on gelatin-coated slides, immediately dried in a stream of air, and exposed to EMC-1 film (Eastman Kodak Co., Rochester, NY, USA) along with calibrated [¹⁴C]methylmethacrylate standards as previously described (Smith and Kang, 2000). Sections were then stained for Nissl substance with Thionin. Autoradiograms and stained sections were digitized with a pixel size of 11 μm by means of a 10-bit DVC-1310 digital camera (DVC, Austin, TX, USA). Images were aligned and analyzed with an MCID Elite image processing system (Imaging Research, St. Catharines, ON, Canada). Regions of interest were located and outlined on the Nissl-stained section by reference to a mouse brain atlas (Paxinos and Franklin, 2001), and optical densities were measured on the autoradiograms. Concentrations of ¹⁴C were determined from the optical density vs. ¹⁴C concentration curve determined from the calibrated plastic standards, and rCMR_{glc} was calculated from the pixel-weighted average local tissue ¹⁴C concentration and the time courses of plasma [¹⁴C]DG and glucose concentrations by means of the operational equation of the method (Sokoloff et al., 1977). We used the rate constants and the lumped constant determined in normoglycemic, conscious rats (Sokoloff et al., 1977). Global cerebral metabolic rate for glucose (CMR_{glc}) and brain volume were determined by analysis of autoradiograms of all sections of the entire brain digitized (42-μm pixel size) by means of a Multirad 850 Howtek Film Digitizer (Howtek Devices, iCAD Inc., Hudson, NH, USA). Weighted average CMR_{glc} was determined as described above, and brain volume was determined from the total number of pixels and the calibrated pixel size.

Statistical analyses

rCMR_{glc} measurements were analyzed for statistically significant differences among the three groups of females by Dunnett's *t*-tests. Corrections for multiple regions compared were made by means of a modified Hochberg procedure with the number of true null distributions estimated by the graphical *p*-plot method; family-wise error was set at α=0.05 (Turkheimer et al., 2001). Physiological variables were analyzed by Dunnett's *t*-tests. Locomotor activity in an open field and acoustic startle response data were analyzed by means of a repeated measures two-way ANOVA. Data sets were further analyzed by post hoc Bonferroni *t*-tests. Latencies determined in the passive avoidance test were analyzed with a one-way ANOVA and post hoc Bonferroni *t*-tests.

Table 1. Physiological variables in female *Fmr1* null mice

	WT (6)	Htz (7)	Hmz (8)
Age (days)	122±1	128±3	123±3
Body weight (g)	19.8±0.4	21.2±0.8	22.5±0.6*
Brain weight (g)	0.47±0.01	0.47±0.07	0.48±0.00
Body temperature (°C)	37.7±0.08	37.9±0.13	37.7±0.07
Hematocrit (%)	44.3±0.8	43.4±0.6	42.6±0.5
Arterial plasma glucose (mM)	8.4±0.6	8.3±0.4	8.6±0.4
Mean arterial blood pressure (mm Hg)	101±3	97±2	98±3

Values are means±SEM for the number of mice indicated in parentheses.

* Statistically significantly different from WT, *P*<0.05, Dunnett's *t*-test.

RESULTS

Regional cerebral metabolic rate for glucose

Physiological measurements were made on the mice in which we measured rCMR_{glc}. Overall the Hmz and Htz *Fmr1* null and WT mice were well matched with respect to physiological variables (Table 1). The only statistically significant difference was a slightly higher (14%) mean body weight in the Hmz *Fmr1* null group compared with WT. Neither CMR_{glc} nor brain volume in Hmz or Htz female *Fmr1* null mice were statistically significantly different compared with WT controls (Table 2). We measured rCMR_{glc} in 53 regions of the brain in the three groups of mice and compared rates in the Hmz and Htz *Fmr1* null mice with WT (Figs. 1 and 2). There were no statistically significant differences between Htz *Fmr1* null mice and WT controls. In comparisons between Hmz *Fmr1* null and WT mice, only the dorsal raphe showed a statistically significant difference (*P*<0.02, Dunnett's *t*-test); rCMR_{glc} in the dorsal raphe was 36% higher in the Hmz *Fmr1* null mice (Fig. 1C). To correct for the effects of multiple comparisons on measurements in 53 brain regions in the same animals we applied the graphical *p*-plot method with the modified Hochberg procedure. This analysis indicates that there is 25% probability that the difference in the dorsal raphe occurred by chance.

Behavioral tests

In contrast to our findings in females we had found that in males with the *Fmr1* null mutation, rCMR_{glc} was significantly increased throughout the brain (Qin et al., 2002). To ascertain whether the different effect of the null mutation in males and females on rCMR_{glc} is due to differential behav-

Table 2. Global CMR_{glc} and brain volume in female *Fmr1* null mice

	WT	Htz	Hmz
CMR _{glc} (μmol/g/min)	0.44±0.03 (5)	0.46±0.03 (7)	0.46±0.03 (7)
Volume (cm ³)	0.460±0.005 (5)	0.451±0.006 (8)	0.470±0.001 (8)

Values are the mean±SEM for the number of mice indicated in parentheses.

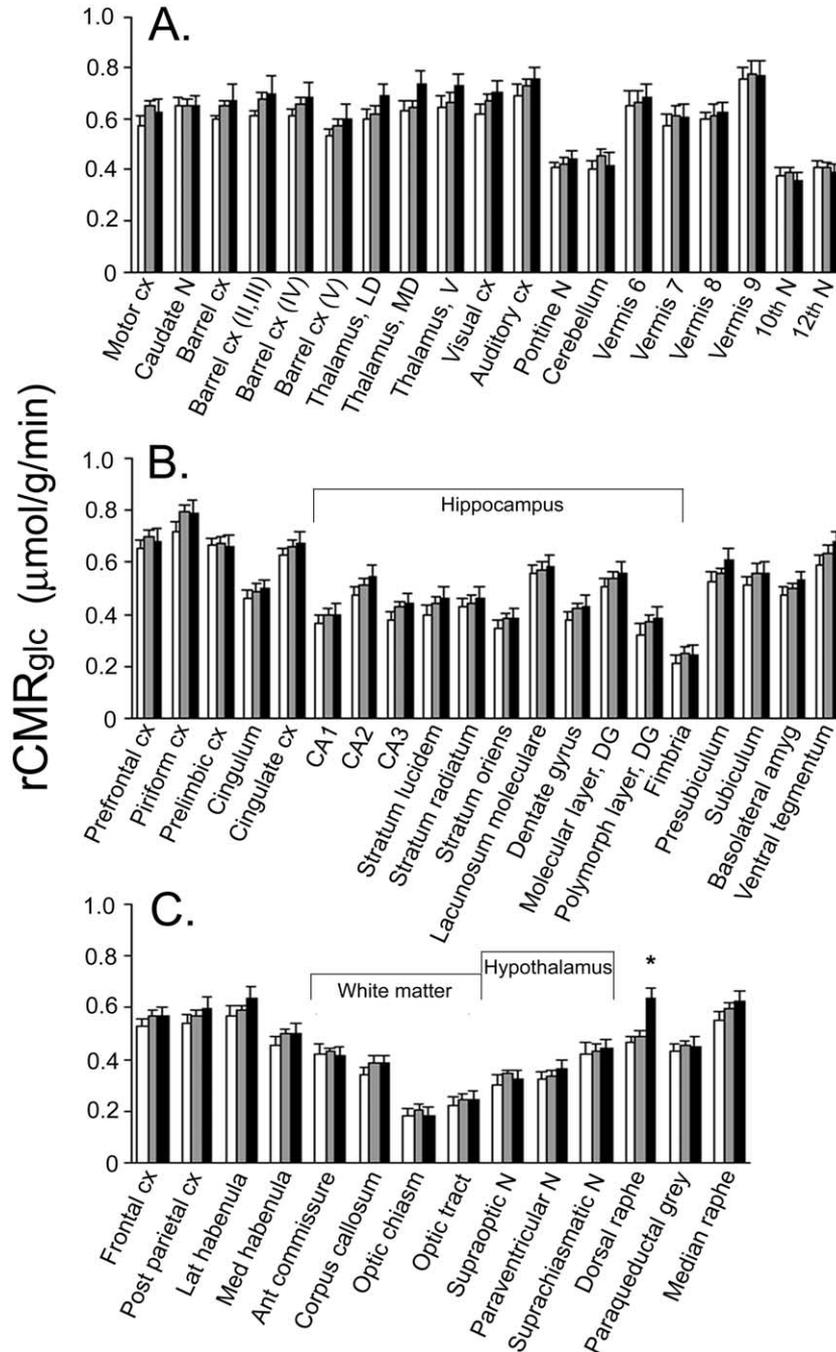


Fig. 1. rCMR_{glc} (μmol/g/min) in 53 brain regions of adult, female *Fmr1* null mice. Values are means±S.E.M. in six WT, seven Htz, and eight Hmz mice; exceptions were SCN and vermis 6 in which *n*=5 for WT, vermis 8 in which *n*=6 for Htz, and optic chiasm and vermis 6 and 7 in which *n*=7 for Hmz. * Statistically significantly different from WT, *P*<0.02, Dunnett's *t*-test.

ioral effects of the mutation we compared male and female *Fmr1* null mice in a series of behavioral tests.

Open-field activity. Results of the open-field test were analyzed by two-way ANOVA (genotype×epoch) with repeated measures on epoch (Fig. 3). We found statistically significant interactions for three of the variables measured (horizontal movement distance (Fig. 3A), % distance moved in the margins (Fig. 3B), and number of

entrances into the center of the field (Fig. 3C)) indicating that the time courses of open field activity differ among the genotypes. The interaction between genotype and epoch was close to statistically significant (*P*=0.074) for the % time spent in the center of the field (Fig. 3D).

Average horizontal distance moved (Fig. 3A) tended to decrease with time in both male and female WT mice and was at a higher level in the WT females in all epochs. At

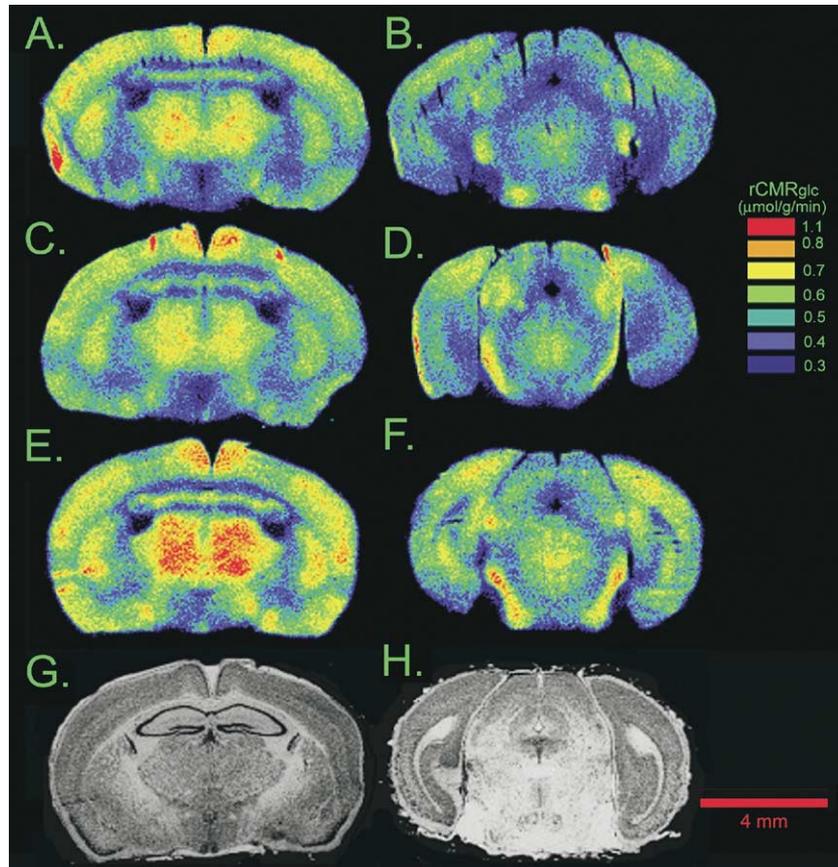


Fig. 2. Digitized [^{14}C]DG autoradiograms (A–F) color coded for rCMR_{glc} and Nissl-stained sections (G, H) corresponding to the autoradiograms E and F, respectively. A and B are from a WT control, C and D are from a Htz *Fmr1* null mouse, and E and F are from a Hmz *Fmr1* null mouse. Images on the left are at the level of the dorsal hippocampus and those on the right are at the level of the dorsal raphe. The scale bar=4 mm in the lower right.

the 6- and 24-min epochs the male–female difference in WT mice was statistically significant ($P < 0.05$, Bonferroni *t*-test). For all three of the *Fmr1* null genotypes average horizontal distance moved tended to be higher than that of both male and female WT across all epochs except the first. In the 18, 24, and 30 min epochs we found statistically significant (post hoc Bonferroni *t*-tests) differences between mutants and same sex WT. Htz and Hmz females moved a greater horizontal distance than WT males at all epochs ($P < 0.05$). There were no statistically significant differences for any epoch between male Hemz and female Hmz mice.

At the 18 and 24 min epochs percent distance moved in the margins of the field (Fig. 3B) tended to be lower for all three *Fmr1* null genotypes compared with both male and female WT, but differences were statistically significant only between WT males and Hemz males. The number of center entrances (Fig. 3C) was similar for WT males and females from 12 to 30 min, and Hemz males had more center entrances than WT males from 18–30 min ($P < 0.05$). Between 18 and 30 min the number of center entrances for Htz and Hmz females tended to be higher than female WT, but these differences were statistically significant only for the Hmz females at the 18 min epoch. Hmz male mice spent an increasing % time in the center of

the field (Fig. 3D) over the course of the test whereas all other genotypes more consistently spent about 30% time in the center.

Passive avoidance. Initially, we tested separate groups of mice for potential differences in pain threshold among the genotypes. All reacted to a 1-s foot-shock at 0.2 mA with vocalizations, jumping, and excessive running demonstrating that they had detected the stimulus. Comparison of the mean latencies to enter the dark chamber for the five genotypes (Fig. 4) by one-way ANOVA shows that there is a statistically significant difference among the means ($F_{(4,121)} = 5.82$; $P < 0.001$). Post hoc Bonferroni *t*-tests show that the mean latency to enter the dark chamber was statistically significantly lower in the male Hemz mice by 58 and 53% compared with either female WT ($P < 0.001$) or Htz mice ($P < 0.01$), respectively and in the female Hmz mice by 37% compared with female WT ($P < 0.05$).

Acoustic startle response. All five genotypes showed increasing startle responses with increasing stimulus intensity (Fig. 5). Responses in the WT males were higher at the higher stimulus intensities compared with all other genotypes, and responses in all other genotypes were very

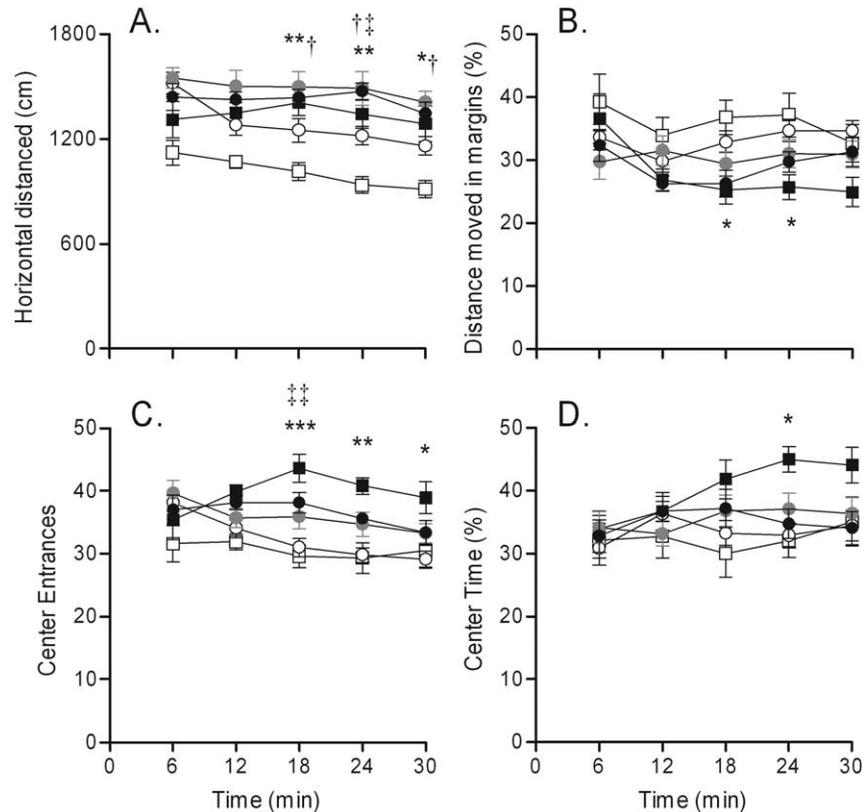


Fig. 3. Open-field activity in WT male (open squares, $n=10$) and female (open circles, $n=20$) mice and Hmz male (black-filled squares, $n=10$), Htz female (gray-filled circles, $n=20$), and Hmz female (black-filled circles, $n=20$) *Fmr1* null mice. Each point represents the mean \pm S.E.M. for each epoch. The data from the male animals were reported previously (Qin et al., 2002), but experiments on all animals of both sexes were carried out at the same time. Data were analyzed by means of a two-way (genotype \times epoch) ANOVA with repeated measures on epoch. The interaction between genotype and epoch for the total distance moved in the horizontal plane (A) was a statistically significant; $F_{(16,300)}=2.17$; $P<0.01$. The interaction between genotype and epoch for the % distance moved in the margins of the field (B) was statistically significant; $F_{(16,300)}=2.39$; $P<0.01$. The interaction between genotype and epoch for the number of entrances into the center of the field (C) was statistically significant; $F_{(16,300)}=2.56$; $P<0.01$. The interaction between genotype and epoch for the % time spent in the center of the field (D) was close to statistically significant; $F_{(16,300)}=1.58$; $P=0.074$. Comparisons among genotypes at each epoch were made by means of post hoc Bonferroni *t*-tests. Statistically significant differences at each epoch between *Fmr1* null mice and same sex WT are indicated on the graphs as follows: * ($P<0.05$); ** ($P<0.01$), *** ($P<0.001$); between male WT and Hmz *Fmr1* null mice. † ($P<0.05$); between female WT and Htz *Fmr1* null mice. ‡ ($P<0.05$); †† ($P<0.01$); between female WT and Hmz *Fmr1* null mice. (A) Horizontal distance moved: between WT male and WT female at the six ($P<0.01$) and 24 ($P<0.05$) min epochs; between WT male and Htz female for all epochs ($P<0.001$); between WT male and Hmz female for 6 ($P<0.05$), 12 ($P<0.01$) min, and all other ($P<0.001$) epochs. (B) % Distance moved in the margins: between WT male and Htz female for the 6 min epoch ($P<0.05$); between WT male and Hmz female for the 18 min epoch ($P<0.05$); between WT female and Hmz male at the 30 min epoch ($P<0.05$). (C) Number of entrances into the center of the field: between WT male and Htz female for the 6 min epoch ($P<0.05$); between WT female and Hmz male at the 18 and 24 min epochs ($P<0.001$) and at the 30 min epoch ($P<0.01$); between Htz female and Hmz male at the 18 min epoch ($P<0.05$). (D) % Time in the center of the field: between WT female and Hmz male at the 24 min epoch ($P<0.05$).

similar to each other at all stimulus intensities. The results of the acoustic startle test for all five genotypes were analyzed by a two-way ANOVA (genotype \times stimulus intensity) with repeated measures on stimulus intensity. The interaction between genotype and stimulus intensity was statistically significant ($F_{24,378}=3.96$, $P<0.0001$). Post hoc Bonferroni *t*-tests indicate statistically significant lower startle responses at the two highest decibel stimuli in Hmz male, Hmz female, Htz female, and WT female compared with WT male mice.

Audiogenic seizures. Animals that responded to the tone usually began with jumping and wild running. In most animals this was followed by myoclonus or tonic seizures and respiratory arrest. About 93% of male and 100% of

female WT mice had no response to the tone (Table 3). In contrast 33% of either Hmz males or Htz females and 40% of Hmz females had some response that in all but one case resulted in a seizure. We used a Fisher's exact test to compare the effects of the mutation on seizure incidence in males and females. Females Htz and Hmz for the mutation were grouped together for this analysis. Results for both sexes were statistically significant ($P<0.05$).

DISCUSSION

Our results demonstrate that there is no statistically significant and widespread effect of the null mutation for *Fmr1* on brain functional activity in females. This contrasts sharply with the noteworthy increases in $rCMR_{glc}$ found

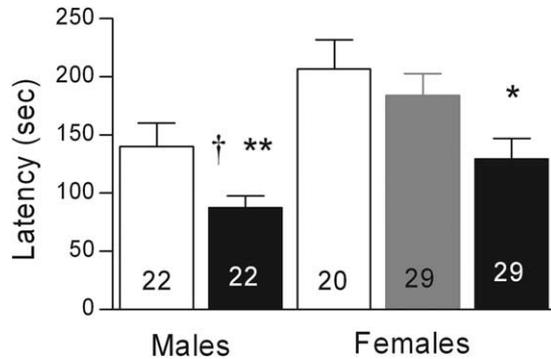


Fig. 4. Passive avoidance task. Bars represent the mean \pm S.E.M. latency to enter the dark chamber 24 h after a single training session in which mice received a foot-shock (0.2 mA for 1 s) when they entered the dark chamber. The number of animals in each group is indicated on the bar. WT are represented by open bars, Hemz and Hmz *Fmr1* null mice are represented by black-filled bars, and Htz *Fmr1* null mice are represented by gray-filled bars. The data from the male animals were reported previously (Qin et al., 2002), but experiments on all animals of both sexes were carried out at the same time so we have analyzed data from both sexes together. Results of one-way ANOVA indicate a statistically significant difference among the means ($F_{(4,121)}=5.82$; $P<0.001$). Statistically significant differences between groups were assessed by post hoc Bonferroni *t*-tests as follows: * $P<0.05$; ** $P<0.001$: statistically significantly different from female WT. † $P<0.01$: statistically significantly different from female Htz *Fmr1* null.

throughout the brains of males with the *Fmr1* null mutation (Qin et al., 2002). Differences in the effect of the *Fmr1* null mutation on behavioral phenotype in males and females were subtle. Both male and female *Fmr1* null mice were hyperactive and susceptible to audiogenic seizures, and both had deficits in performance on a simple test of learning and memory. Male *Fmr1* null mice showed evidence of

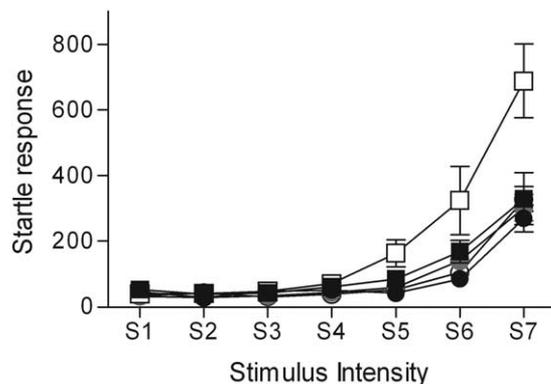


Fig. 5. Acoustic startle response in WT male (open squares, $n=14$) and female (open circles, $n=14$) mice and Hemz male (black-filled squares, $n=18$), Htz female (gray-filled circles, $n=12$), and Hmz female (black-filled circles, $n=10$) *Fmr1* null mice. Each point represents the group mean \pm S.E.M. Background noise was 60 dB. Stimulus intensities were 0 (S1), 70 (S2), 80 (S3), 90 (S4), 100 (S5), 110 (S6), 120 (S7) dB. Results were analyzed by means of two-way (stimulus intensity \times genotype) ANOVA with repeated measures on stimulus intensity. The interaction between stimulus intensity and genotype was statistically significant ($F_{24,378}=3.96$, $P<0.0001$). By post hoc analysis there were statistically significant differences ($P<0.01$) between responses of WT male and all other groups at S6 and S7.

Table 3. Audiogenic seizures in *Fmr1* null mice

Genotype	N	No response	Seizure
Male WT	14	13	0
Male <i>Fmr1</i> null	18	12	6
Female WT	14	14	0
Female <i>Fmr1</i> null Htz	12	8	3
Female <i>Fmr1</i> null Hmz	10	6	4

Mice were acclimated to the chamber for 5 min and then subjected to 1 min of 120 dB generated by a doorbell. *N* is the number of mice tested. In response to the bell one male WT mouse exhibited jumping and one female Htz *Fmr1* null mice exhibited both wild running and jumping but no other response. Mice were 143 ± 13 (mean \pm SD) days of age.

decreased anxiety in the open field whereas females did not, and in *Fmr1* null males acoustic startle responses were diminished at higher intensity stimuli whereas in females responses were similar to those in female WT.

Functional activity

We applied the autoradiographic [14 C]DG method to determine $rCMR_{glc}$ as a measure of regional functional activity. The method was originally devised for use in the rat (Sokoloff et al., 1977) but has been applied to many other species (Sokoloff, 1996). The practical considerations for the adaptation of the method for use in the mouse have been discussed elsewhere (Qin et al., 2002). The values we report here for $rCMR_{glc}$ in female WT controls are in good agreement with values in male WT controls on the same (FVB/NJ) background reported previously (Qin et al., 2002) indicating that as was shown in the rat (Nehlig et al., 1985) there is little if any effect of sex on $rCMR_{glc}$.

We did not control for the stage of the estrous cycle in our study. In rats there is no overall effect of the stage of the estrous cycle on global CMR_{glc} but there are some discrete changes in regions of hypothalamus and limbic system (Nehlig et al., 1985). At the time of the Nehlig study there were no powerful statistical methods available for controlling for false positives in data sets with multiple regions in the same subjects, and the authors reported their results without any such correction. We have reanalyzed their data with the *p*-plot method and we find that regions with statistically significant effects due to stages of the estrous cycle are localized to the medial preoptic area of the hypothalamus and superior colliculus. Our present study did not include these two regions. We did observe higher variance in our measurements of $rCMR_{glc}$ in female *Fmr1* null mice than in either WT or *Fmr1* null males, but variance in WT females was similar to that of males (Fig. 6) supporting the idea that the higher variance is an effect of genotype rather than sex. We also looked at the variances for an effect of mosaicism with the expectation that mosaicism might produce higher variance among Htz females. Variances in Htz and Hmz *Fmr1* null females, however, were similar except in the dorsal raphe (Fig. 6H) in which the variance in the Hmz mice was greater than that in the Htz mice suggesting that we are not observing the effects of mosaicism.

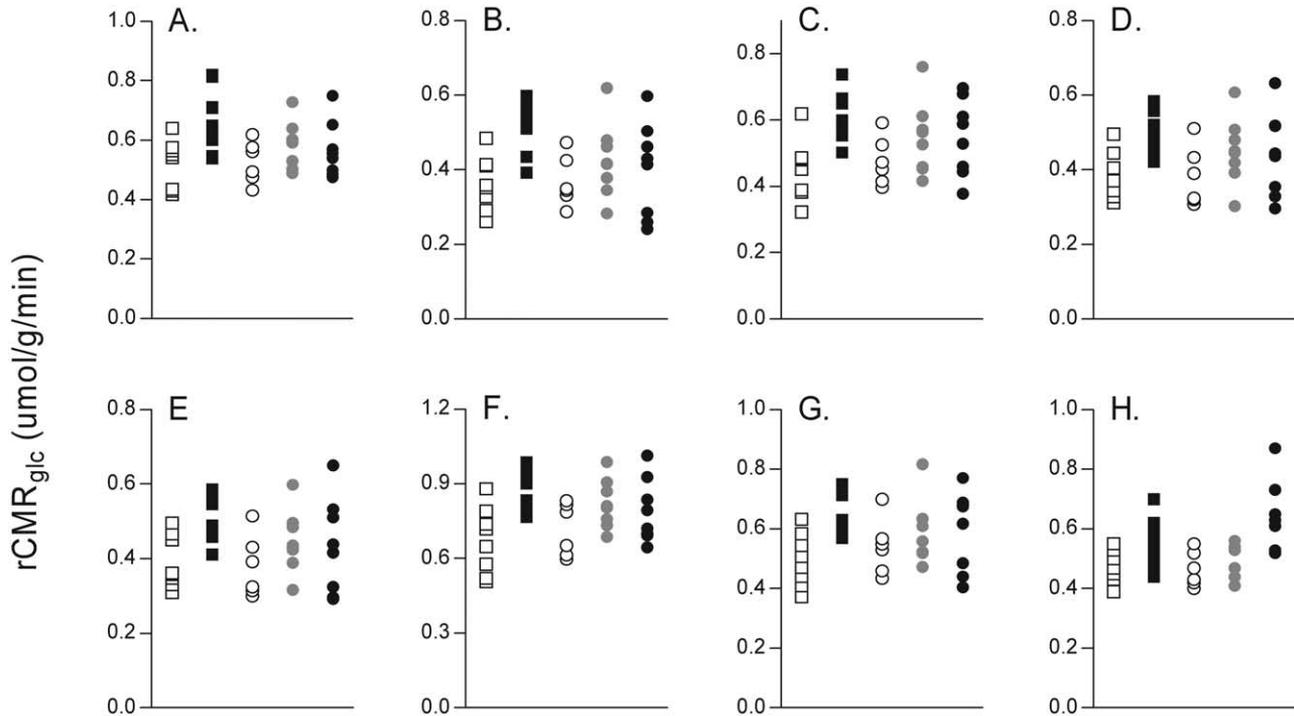


Fig. 6. Individual values for $rCMR_{glc}$ in frontal association cortex (A), pyramidal cell layer of the dorsal hippocampus CA1 sector (B), CA2 sector (C), CA3 sector (D), granule cell layer of the dentate gyrus (E), piriform cortex (F), posterior parietal cortex (G), and dorsal raphe (H). Open, gray and black circles represent values from female WT, Htz and Hmz *Fmr1* null mice, respectively; open and black squares represent values from male WT and Hmz *Fmr1* null mice, respectively. Effects of the mutation were statistically significant in males in all regions (A–G, $P < 0.01$; H, $P < 0.05$; Student's *t*-test) (Qin et al., 2002) and in females only in the dorsal raphe ($P < 0.02$, Dunnett's *t*-tests).

In contrast to our findings in male *Fmr1* null mice in which $rCMR_{glc}$ was considerably increased throughout the brain (Qin et al., 2002), in female *Fmr1* null mice CMR_{glc} in the brain as a whole and in most of the brain regions analyzed was not different from that of WT controls. This was true for both Htz and Hmz female *Fmr1* null mice. The one brain region in which we found statistically significantly increased $rCMR_{glc}$ in the female Hmz *Fmr1* null mice is the dorsal raphe. According to the results of the *p*-plot analysis which corrects for multiple comparisons this effect may have occurred by chance. It is interesting however that there is very good agreement between values of $rCMR_{glc}$ in the dorsal raphe among Htz females and WT of both sexes (Fig. 6H). Values of $rCMR_{glc}$ in Hmz male and Hmz female *Fmr1* null mice are more variable and generally higher than those in the other three groups, and values in the Hmz female *Fmr1* null mice tend to be higher than those in the *Fmr1* null males. In our present study mean $rCMR_{glc}$ in dorsal raphe is increased by 36% in female *Fmr1* null mice compared with WT. In males the increase in *Fmr1* mice was 19% ($P < 0.05$) (Qin et al., 2002).

An effect on the dorsal raphe is of interest in the light of the hyperactivity associated with FraX and the possible role of serotonergic projections from the raphe nuclei in hyperactivity (Tao and Auerbach, 2003; Kusljic et al., 2003). Serotonergic projections from the dorsal raphe terminate in frontal cortex, ventral hippocampus, and striatum (Adell and Myers, 1995). We did not analyze ventral hippocampus in our studies, but in *Fmr1* null males we found

a statistically significant increase (30%) in $rCMR_{glc}$ in frontal cortex but not in caudate-putamen (Qin et al., 2002). In females neither region had an increased $rCMR_{glc}$. Since effects on $rCMR_{glc}$ usually reflect synaptic activity (Sokoloff, 1996) dorsal raphe afferents are an important consideration. The major afferents are from the raphe nuclei themselves. Other inputs arise from the superior vestibular, nucleus prepositus hypoglossi, perihypoglossi, nucleus of the solitary tract, locus coeruleus, substantia nigra, ventral tegmental area, and periaqueductal gray. The most important hypothalamic inputs come from the medial preoptic area and lateral hypothalamus. Of these regions we analyzed median raphe, ventral tegmentum and periaqueductal gray in our studies of male and female *Fmr1* null mice and we found no effects on $rCMR_{glc}$. We also found no effects in the hypoglossal nuclei. Of the efferents of the dorsal raphe in which we measured $rCMR_{glc}$ only frontal cortex is affected and only in the male *Fmr1* null mice. Of the afferents that we measured none was affected in either male or female *Fmr1* null mice.

The increased $rCMR_{glc}$ that we observed in the dorsal raphe could reflect an increase in spontaneous neural activity. The serotonergic neurons of the dorsal raphe are spontaneously active, and the balance between GABAergic and glutamatergic input may modulate their discharge rate. Studies in rats have shown that reverse dialysis infusion of bicuculline, a GABA_A receptor antagonist, into the dorsal raphe increased locomotion (Tao and Auerbach, 2003). As in the cortex where dendritic abnormalities in

FraX patients may be responsible for unbalanced excitatory inputs causing hyper-excitability, serotonergic neurons in the dorsal raphe may discharge at an increased rate due to a similar unbalance. The increased rCMR_{glic} may reflect such an unbalanced modulatory input.

Behavioral assessment

The lack of an effect of the *Fmr1* null mutation on rCMR_{glic} in female mice was unexpected and we hypothesized that it might be related to a male–female difference in the behavioral phenotype. Male *Fmr1* null mice are hyperactive in an open field (Bakker et al., 1994; Peier et al., 2000; Mineur et al., 2002; Qin et al., 2002), and it is reasonable to conjecture that hyperactivity might be associated with a generalized increase in functional activity in the brain as seen in the male *Fmr1* null mice (Qin et al., 2002). Since we had tested the male and female mice at the same time we reanalyzed the male data with the female data to test for differences in open field behavior among the five genotypes. Results indicate that all animals with the *Fmr1* null mutation were hyperactive regardless of sex, and heterozygosity in the females did not appear to diminish the effect.

We analyzed relative distance moved in the margins of the field, the number of entrances into the center of the field, and the percent time spent in the center of the field as indices of anxiety (Crawley, 1989). By these criteria male *Fmr1* null mice exhibited reduced anxiety-related behavior compared with WT, but in females this effect is not so clear. Observations of hyperactivity and diminished anxiety-related behavior in an open field have also been reported in male *Fmr1* null mice on a C57BL/6 background (Peier et al., 2000). Peier et al. (2000) demonstrated that these characteristics were reversed in *FMR1* yeast artificial chromosome (YAC) transgenic mice. Since we found hyperactivity in both male and female *Fmr1* null mice the differential effect of the null mutation on rCMR_{glic} cannot be attributed to hyperactivity. Further, we would have predicted that diminished anxiety as seen in male *Fmr1* null mice would be associated with a decreased rCMR_{glic} since treatment with anxiolytic drugs such as diazepam reduces rCMR_{glic} in rats (Eintrei et al., 1999).

We hypothesized that the male–female difference in the effect of the *Fmr1* null mutation on rCMR_{glic} may be due to a difference in sensitivity to sensory stimuli. Patients with FraX have an increased sensitivity to sensory stimuli (Miller et al., 1999; Hagerman, 2002). We measured the acoustic startle response in male and female *Fmr1* null mice to test for differences in auditory responses and found that the stimulus intensity–response curve for the WT male mice is at a higher level compared with the other four genotypes. Compared with male WT the *Fmr1* null mutation in males results in lower responses particularly at the higher decibel stimuli (110 and 120 dB), but responses in all three female genotypes were similar to those of the *Fmr1* null male. It is of interest that the startle response tends to be higher with increased anxiety (Koch, 1999), and Hemz male *Fmr1* null mice exhibited reduced anxiety-like behavior in the open field test.

The male–female difference for the WT mice is supported by the auditory startle response data in rats in which males tended to have a higher response magnitude at 100 dB than females (Koch, 1998). Our results at higher intensity stimuli in males agree with the findings in *Fmr1* null mice on the same background (Chen and Toth, 2001), on a C57 background and in an F1 (C57BL/6J×FVB) hybrid (Nielsen et al., 2002). In the C57 and F1 hybrids, however, small but statistically significant increases in startle response were also seen at very low intensity stimuli (80 dB) (Nielsen et al., 2002). Our *Fmr1* null mice did not show any evidence of increased response at any stimulus intensity. This difference could be a function of genetic background or age. In the study of Chen and Toth (2001) startle response was measured in FVB/NJ mice at 7–10 weeks of age but only at a stimulus intensity of 115 dB. In the study of Nielsen et al. (2002) C57 mice and F1 hybrids were studied at 14–16 and 8–9 weeks of age, respectively. We tested our FVB/NJ mice at 17–23 weeks, the age at which we measured rCMR_{glic}; and they did not respond at all to the low intensity stimuli. The acoustic startle thresholds in our FVB/NJ males and females were 100 and 110 dB, respectively. In the C57 and F1 hybrids startle responses were observed at 80 dB (Nielsen et al., 2002). A diminished hearing acuity in our older mice may explain this difference in thresholds. In view of the report that in younger *Fmr1* null mice the startle response at lower intensities is elevated and at higher intensities is diminished, the reduced startle response at higher stimulus intensities found in our male *Fmr1* null mice is likely due to defects in the pathways that influence the startle response rather than a primary sensory disturbance.

Susceptibility to seizures particularly during childhood is another characteristic of FraX that might be associated with hyperexcitability. We compared susceptibility to audiogenic seizures in male and female *Fmr1* null mice to see if a difference in susceptibility could help us to understand the male–female difference in the effect of the null mutation on rCMR_{glic}. Our study shows that both adult male and female *Fmr1* null mice have increased susceptibility to audiogenic seizures compared with WT. FVB mice are known to be seizure prone (Goelz et al., 1998), but the incidence of audiogenic seizures in *Fmr1* null mice on the FVB background was higher than WT. Our results agree with two other studies of fragile X mice that showed higher incidences of audiogenic seizures in Hemz male and Hmz female *Fmr1* null mice at earlier stages of development (Musumeci et al., 2000) and in adult male *Fmr1* null mice (Chen and Toth, 2001). Despite the male–female differences in the postnatal development of susceptibility to audiogenic seizures seen in *Fmr1* null mice (Musumeci et al., 2000) by adulthood susceptibilities were similar. The incidence of audiogenic seizures (70%) reported in male *Fmr1* null mice between 140 and 238 days of age was considerably higher (Chen and Toth, 2001) than the incidence (33%) we observed in 120–150 day old mice. The stimulus (white noise, 2–20 kHz) in our study was very similar to that used in the Chen and Toth (2001) study although our stimulus intensity was slightly higher (120 v.

115 dB). Probably the difference in age-ranges of the animals is the significant difference between the studies, suggesting that susceptibility may continue to change with age even in adult mice.

The primary symptom of FraX is mental retardation. It has been suggested that the excess of immature dendritic spines found in neocortex in FraX might be related to an inability to stabilize synaptic contacts which leads to diminished cognitive ability. The immature spines and unstabilized synapses may result in hyperexcitability which in turn may be responsible for the increase in cerebral energy metabolism seen in the male *Fmr1* null mouse (Qin et al., 2002). In our previous study we reported that male *Fmr1* null mice were significantly impaired on the passive avoidance test of learning and memory (Qin et al., 2002). We also tested female mice on the same test and analysis of the results in all five genotypes together shows a clear deficit on the passive avoidance test in both male *Fmr1* null and female Hmz mice compared with female WT. Females Htz for the *Fmr1* null mutation were not affected, suggesting that the impairment only occurs in the complete absence of FMRP.

Based on the similarities between male and female *Fmr1* null mice we posit that increased rCMR_{glc} in the males is not merely a function of hyperactivity and increased susceptibility to seizures and is not related to a deficit in performance on the passive avoidance test. There are several differences between males and females in the behavioral manifestation of the *Fmr1* null mutation. Male mice showed a diminution of anxiety-related behavior and a decreased startle response compared with WT whereas females do not. Based on the increased rCMR_{glc} found in male *Fmr1* null mice we would have predicted behavioral effects in the opposite direction, i.e. increased anxiety and increased startle response. It is possible that these particular behavioral traits have no bearing on the changes in rCMR_{glc}. Alternatively, the apparent mismatch may be a function of the state (resting) in which we measured rCMR_{glc}. Measurement of rCMR_{glc} in the animals during an anxiety-provoking event or during sensory stimulation might produce a very different picture. In the “resting” animal functional activity in the nervous system is not affected by the *Fmr1* null mutation in females whereas it is statistically significantly increased in males. Whether or not estrogen affords female *Fmr1* null mice some protection from the increased functional activity in brain warrants further investigation.

Acknowledgments—We thank T. Burlin for help with processing the autoradiographs. This research was supported by the Intramural Research Program of the NIMH, NIH.

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(Accepted 16 June 2005)
(Available online 8 September 2005)