Neuroscience 135 (2005) 999–1009

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.

0306-4522/05$30.00+0.00 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.06.081

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 (Hemz) *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: Hmz 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′-GTGGTTAGCTAAAGTGAGGATGAT-3′) and S2 (5′-CAGGTTTGTTGGGATTAACAGATC-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 photobeams 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 Vmax (the peak response in the 150 ms) was used as the stimulus response level. For each animal at each stimulus intensity the mean Vmax 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