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A phenotypic and molecular characterization of the *fmr1-tm1Cgr* Fragile X mouse

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Fragile X Syndrome is the most common form of inherited mental retardation. It is also known for having a substantial behavioral morbidity, including autistic features. In humans, Fragile X Syndrome is almost always caused by inactivation of the X-linked *FMR1* gene. A single knockout mouse model, *fmr1-tm1Cgr*, exists. In this report we further characterize the cognitive and behavioral phenotype of the *fmr1-tm1Cgr* Fragile X mouse through the use of F1 hybrid mice derived from two inbred strains (FVB/NJ and C57BL/6J). Use of F1 hybrids allows focus on the effects of the *fmr1-tm1Cgr* allele with reduced influence from recessive alleles present in the parental inbred strains. We find that the cognitive phenotype of *fmr1-tm1Cgr* mice, including measures of working memory and learning set formation that are known to be seriously impacted in humans with Fragile X Syndrome, are essentially normal. Further testing of inbred strains supports this conclusion. Thus, any *fmr1-tm1Cgr* cognitive deficit is surprisingly mild or absent. There is, however, clear support presented for a robust audiogenic seizure phenotype in all strains tested, as well as increased entries into the center of an open field. Finally, a molecular examination of the *fmr1-tm1Cgr* mouse shows that, contrary to common belief, it is not a molecular null. Implications of this finding for interpretation of the phenotype are discussed.

Keywords: Audiogenic seizure, Barnes maze, *Fmr1* RNA, Fragile X, hybrid mouse strain, Morris water maze, olfactory sequence learning, open field, radial maze, working memory

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Fragile X Syndrome (FXS) is a genetic disorder characterized by mental retardation, behavioral problems and physical manifestations, most notably macroorchidism (Escalante *et al.* 1971; Martin & Bell 1943). It is the most common form of inherited mental retardation, with a prevalence of approximately 1 in 4000 males (Morton *et al.* 1997; Turner *et al.* 1996). Those with FXS lack the protein FMRP (Fragile X Mental Retardation Protein) in neurons. FMRP is widely expressed in vertebrate tissues, particularly in those with dividing cells such as the developing brain (Devys *et al.* 1993; Khandjian *et al.* 1995). In almost all cases of FXS, the absence of FMRP is usually associated with a triplet repeat expansion in the 5′ UTR of the X-linked *FMR1* gene which leads to promoter methylation and transcriptional silencing (Bell *et al.* 1991; Sutcliffe *et al.* 1992).

FMRP is an RNA binding protein (Siomi *et al.* 1993) associated with approximately 4% of brain mRNA (Ashley *et al.* 1993) and has been observed on ribosomes in the neuronal soma and dendrites (Feng *et al.* 1997; Weiler & Greenough 1999). FMRP has the molecular characteristics of an mRNA nuclear–cytoplasmic shuttling protein, and can inhibit translation of mRNAs (Laggerbauer *et al.* 2001; Li *et al.* 2001; Zalfa *et al.* 2003). The presence of FMRP in dendrites, particularly at the base of spines, suggests that it is involved in mRNA targeting to synapses and/or the translational regulation of such mRNAs. Since dendritic abnormalities have been observed in FXS, it is possible that FMRP is involved in synaptic development and plasticity.

Ideally, animal models of Fragile X Syndrome should exhibit the hallmark behavioral and cognitive deficits observed in affected humans. Infants with Fragile X are usually noted to nurse poorly and are late in achieving developmental milestones such as sitting up. Hand flapping, poor eye contact and other autistic features are characteristically seen by 5 years of age. Perseveration in speech and behavior are frequently very pronounced, as are attention deficits, impulsivity and hyperactivity (Baumgardner *et al.* 1995; Bregman *et al.* 1988; Fisch *et al.* 1999a; Munir *et al.* 2000b; Wilding *et al.* 2002); over 70% of children with Fragile X have a diagnosis of attention deficit hyperactivity disorder (ADHD) (Hagerman 1996). Hyperarousal and hypersensitivity to sensory stimuli are notable in FXS, as is the frequency of childhood seizures (more than 15%; Musumeci *et al.* 1999). Anxiety, particularly to novel situations, can also be a major feature of FXS. The mean IQ of males with FXS is 35–40 (reviewed in de la Cruz 1985). However, cognitive impairment in FXS is quite variable, and is most likely correlated

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with the degree of tissue and methylation mosaicism (Kaufmann *et al.* 1999). In IQ tests, males with FXS show a relative deficit in sequential task processing, consistent with a deficit in short-term/working memory (Bennetto & Pennington 1996; Dykens *et al.* 1987).

In order to further investigate Fragile X Syndrome and its effect on intelligence, genetically manipulable animal models are essential. To this end, a knockout (KO) mouse has been produced by insertion of a selectable marker sequence into the fifth exon of the *Fmr1* gene, which has 17 exons, thereby apparently disrupting transcription and translation of most of the gene lying-3′ of exon 5 (Dutch–Belgian Fragile-X Consortium 1994); a function for exon 5 itself is not known. The *fmr1-tm1Cgr* KO mouse shares morphologic features of FXS in humans, such as macroorchidism (Dutch–Belgian Fragile-X Consortium 1994) and neuronal dendritic spine abnormalities (Comery *et al.* 1997; Nimchinsky *et al.* 2001). Auditory seizures can be induced in *fmr1-tm1Cgr* mice as well (Chen & Toth 2001; Musumeci *et al.* 2000). Although no alterations in *fmr1-tm1Cgr* hippocampal long-term potentiation (LTP) have been found (Godfraind *et al.* 1996; Paradee *et al.* 1999; Valentine *et al.* 2000), a deficit in cortical LTP has been described (Li *et al.* 2002), as has an elevation in mGluR group I dependent hippocampal LTD, which may be associated with elevated AMPA receptor internalization (Huber *et al.* 2002).

Prior reports describing the behavioral phenotype of the *fmr1-tm1Cgr* mouse have produced inconsistent results, and even behaviors seemingly the opposite of what would be expected from the human syndrome. For example, in spatial memory tests employing the Morris water maze (MWM), some groups observed possible mild deficits (D'Hooge *et al.* 1997; Dutch–Belgian Fragile-X Consortium 1994; Kooy *et al.* 1996) while others saw no difference between mutant and wildtype (WT) animals which could be ascribed to the *fmr1-tm1Cgr* allele (Paradee *et al.* 1999; Peier *et al.* 2000). Likewise, differences from WT animals were found in a plus maze in one study (Van Dam *et al.* 2000) but not in a second (Dobkin *et al.* 2000). Locomotor hyperactivity in the mutant animals has been reported (Dutch–Belgian Fragile-X Consortium 1994) as has its absence (Nielsen *et al.* 2002; Peier *et al.* 2000). In one study, *fmr1-tm1Cgr* mice performed significantly better than WT in operant learning and memory tasks (Fisch *et al.* 1999b). Oddly, some of the more reproducible findings, principally a seemingly reduced anxiety in various tests (Peier *et al.* 2000), and a diminished response to audiogenic startle (Chen & Toth 2001; Nielsen *et al.* 2002), suggest a phenotype the opposite of that observed in humans with FXS (Bregman *et al.* 1988; Hagerman 1996; Miller *et al.* 1999).

Here we describe further cognitive and behavioral tests of *fmr1-tm1Cgr* mice. Original test results are presented in several assays of working memory: novel object, radial maze and olfactory sequence. Results from testing in the Barnes maze, assessing spatial long-term memory, are also reported for the first time in *fmr1-tm1Cgr* mice. In addition, Barnes maze behaviors were compared to results from the MWM. Overall, the cognitive effects of the *fmr1-tm1Cgr* mutation were surprisingly mild or undetectable. Tests of hyperarousal, including open field, elevated plus maze, acoustic startle and audiogenic seizure susceptibility were also performed. *fmr1-tm1Cgr* mice showed significant alterations in open field behavior and audiogenic seizure sensitivity. Finally, the molecular phenotype of the *fmr1-tm1Cgr* mouse was further characterized. The *fmr1-tm1Cgr* mouse is shown to express substantial *fmr1* mRNA, including one isoform which has an open reading frame (ORF) extending the length of the gene and which would be expected to produce a full length FMRP. Implications for the phenotypes are discussed.

Materials and methods

Subjects

FVB/NJ (FVB), C57BL/6J (C57) and mice with the *fmr1-tm1Cgr* ('*fmr1*') allele on the FVB and C57 backgrounds (strain numbers 001800, 000664, 003024 and 003025, respectively) were obtained from Jackson Laboratories (Bar Harbor, MN). The *fmr1-tm1Cgr* mouse has been referred to as a 'knockout' (KO) in almost all prior reports; this designation will also be used in this study, although a more exact description of the molecular phenotype indicates that the allele is not a null (see *Results*). Male C57BL/6J *fmr1-tm1Cgr* mice were bred to WT C57BL/6J mice to produce females heterozygous for the *fmr1* mutant allele. Male FVB/NJ mice were bred to heterozygous C57BL/6J+/*fmr1-tm1Cgr* female mice in order to produce litters with approximately half WT and half *fmr1* mutant ('KO') males. Mice of the opposite strain cross (C57 × FVB het) were also used, as indicated ('CxF'). The FxC and CxF F1 hybrid mice are denoted 'hybrid' or 'HYB' throughout the text. Animals were always on an F1 hybrid background unless specifically noted otherwise. Mice were weaned no earlier than 3.5 weeks of age except in audiogenic seizure priming experiments for C57 and hybrid strains. At weaning, tail and toe clippings were taken for genotypic identification of the animals. Females, and noticeably runted mice, if any, were discarded and the males housed in groups of up to five. Adult males were housed individually while undergoing the olfactory and strong choice radial maze tasks. Animals were first tested between three and five months of age, unless noted otherwise (e.g. for the audiogenic seizures, below). A 12:12 h light/dark cycle was employed at all three sites, with testing in the light phase at two (Columbia and Sention) and in the dark phase at one (Dalhousie). Food (e.g. PicoLab Mouse Diet 20, Columbia) and water were *ad libitum* except for the radial maze and olfactory working memory tests (see below).

Ten WT and 14 KO mice were shipped to Dalhousie University where they were kept in quarantine for two weeks and then tested in a battery of behavioral tests (Brown *et al.* 2000) which measured anxiety and locomotor activity (elevated plus maze, open field), non-spatial memory (novel object recognition) and spatial memory (Barnes maze, MWM, 8-arm radial maze) in that order. Twelve WT and KO mice each were used in the startle and PPI tests (Sention). Nine WT and 10 KO mice were used for the olfactory working memory and second radial maze tests (Columbia). All of the preceding were F1 hybrid mice. Eleven FVB, 20 FVB KO, 13 C57, 6 C57 KO, 33 hybrid and 8 hybrid KO mice were used for audiogenic seizure testing (Columbia).

Genotyping and molecular analysis

Mice were genotyped by PCR for the presence of the neomycin ('neo') antibiotic resistance sequence found at the *fmr1-tm1Cgr* disruption site (primer sequences are given in Table 2). Mice were also genotyped for the presence of the WT *Fmr1* allele using the exon 5 primers Ex5(5′)/(3′) spanning the neo insert. PCR conditions were typically 1 cycle of 94 °C, 4 min, 30–35 cycles of 94 °C, 30 seconds/annealing temperature 'Ta' (Table 2), 1 min/72 °C, 2 min, and a final extension of 72 °C, 7 min, on a Perkin-Elmer 2400 thermal cycler (Wellesley, MA). Generally, 0.25 unit/25 μl reaction Taq polymerase (Promega, Madison, WI, with Buffer B) was used, along with 200 μM final dNTPS, and 0.2–1 μM final primers.

Total RNA and cDNA was made from brain, growing tail tips, white blood cells, and other organs as previously described (Bauchwitz & Costantini 2000). Two μg of cDNA was routinely used for RT-PCR. Five μg of oligo-dT selected polyadenylated RNA was used for Northern blots (Fig. 9) or 16 μg of total RNA (not shown). Antisense ^{32}P labeled RNA probes for Northern blotting were made from PCR products in which one primer included a terminal T7 RNA polymerase phage promoter sequence. Modified nucleotides were incorporated into the labeled RNA probes which allowed more efficient removal from the blots (Ambion Inc. Strip-EZ, Austin, TX). The *Fmr1* specific probe used in Fig. 9 spanned the 3′-UTR region bounded by the MuSp primers (Table 2), a region with no significant overlap to murine *FXR* sequences or others found by BLAST database seaches. RNA was separated on 1% agarose gels using BPTE buffer (Burnett 1997). Gels were blotted by downward transfer in 10 × SSC to positively charged nylon membranes. Northern blots were hybridized at 63 °C in 50% formamide buffer which in some cases (Fig. 9) included a volume excluder (Cornish *et al.* 1998; Ambion UltraHyb). Blots were washed at 63 °C twice for 5 min with 2xSSC/0.1%SDS followed by 63 °C twice for 15 min with 0.1xSSC/0.1%SDS.

RT-PCR across the *fmr1-tm1Cgr* insertion site (e.g. Fig. 9) was as described for genotyping except that cycle extensions were usually increased to 3 min. RT-PCR products from a primary PCR were diluted as necessary (1/12–1/50 final) for a second round of PCR, as indicated in the text. A nested PCR protocol was used to specifically identify the full length ORF isoform of *fmr1-tm1Cgr* (Fig. 10). cDNA was made from 1 μg of each RNA sample with a one step method (One-Step RT-PCR for Long Templates, Invitrogen, Carlsbad, CA; 55 °C, 30 min) and 0.5 μM *Fmr1* exon 3 and exon 7 primers (1 cycle of 95 °C × 5 m followed by 40 cycles of 95 °C × 1 m, 54 °C × 2 m, 68 °C × 3 m). Subsequently, 2 μl of this product was used in a 50 μl PCR reaction with 3neoiso – U1/L, U2/L or U3/L primers. The 3neoiso-U1 upstream primer was a nested *Fmr1* exon 3 primer. An upstream primer which crossed a novel junction of the full length *fmr1-tm1Cgr* isoform was also used (3neoiso-U2; 5′-TGATAAAGGGTGAGATCATC) with the same 3neoiso-L primer (Table 2) to produce a 187 bp KO specific PCR product ('U2'). Sensitivity of the 3neoiso-U2 primers and others spanning the U2187 bp product splice junctions (U4, U5 and U6, each with 3neoiso-L, unpublished data) was low as assessed by titration of known amounts of sequenced 187 bp product in PCR reactions. Therefore, 'touchdown' cycling protocols, with and without 1.2 M betaine, were also employed when using U2/L primers as a primary screen, e.g. 95 °C × 4 m; 5 cycles of 94 °C × 30 seconds, 72 °C × 45 seconds, 72 °C × 1 : 00 min, for which the annealing temperature was decreased by 1.0 °C each cycle; 35 cycles of 94 °C × 30 seconds, 67 °C × 45 seconds, 72 °C × 1 min for which the annealing temperature was decreased by 0.2 °C each cycle; 72 °C.

RNase protection analysis was used to estimate the quantity of splice products in adult mouse brains using the U2 splice junctions. A 208 nucleotide (NT) probe template was made by PCR between the 3neoiso-U2 primer and a T7 promoter sequence followed by a spacer (5′TAATACGACTCACTATAGGATTTAGGTGACACTATAGAGATAAGCTTGATATCGAATTCTTG). The probe was labeled with ^{32}P-UTP 800 Ci/mmole, 10 mCi/ml, in an *in vitro* transcription (Ambion MaxiScript) and then gel purified on a 15% acrylamide urea denaturing gel. 50 μg of total RNA from adult KO and WT mice, as well as from yeast as a negative control, were processed as described (Ambion RPAIII; Fermentas RNaseA/T1 mix, 1:50). Samples and labeled markers (Century Plus, Ambion) were separated for 90 min at 300 V on a 15% acrylamide denaturing gel. Template RNA for a standard curve was produced by PCR between the 3neoiso-L primer and an oligonucleotide with a T7 promoter sequence upstream of the U2 primer sequence. A common band was observed in all RNase protection ('RPA') samples, including those in which the probe was hybridized to yeast total RNA (see Fig. 10a). The unknown common band was resistant to twice the RNase concentrations normally used in this procedure. This RNA is shorter than a full length probe, and may be a self-protected species, perhaps reflecting significant secondary structure.

Sequence analysis was used to confirm the identity of the 3neoiso-U1 and U2 products, as well as a third splice isoform from KO mice which employed a different 5′ splice junction (*fmr1* exon 4 to bovine pA): 3neoiso-U3; 5′GGGTGAGGAAAGGACAG.

The 3neosio-U3/L primer pair produced a 178 bp product (Ta = 56°; Fig. 10). The U1 and U3 PCRs were performed with a hotstart method (1.25 μ./50 μl reaction AmpliTaq Gold, Applied Biosystems, Foster City, CA) 95 °C, 10 m, 35 cycles of 95 °C, 30 seconds, Ta 1 m, 72 °C, 2 m. Ta U1 = 54 °C; Ta U3 = 56 °C.

Elevated plus maze

The elevated plus maze (EPM) was built according to the description of Lister (Lister 1987) and was illuminated with a 60-watt red light. Mice were tested for 5 min and their behavior videotaped. Behaviors scored included: (1) frequency of entering the open arms with all four paws, (2) frequency of entering the closed arms, (3) time spent in the open arms, (4) time spent in the closed arms, (5) frequency of entering the central square with all four paws, (6) time spent in the central square, (7) frequency of head dipping over the sides of the open arm, (8) frequency of stretch attend postures, (9) frequency of rearing, (10) frequency of nonexploratory behavior (grooming or not moving), (11) number of puddles or streaks of urine, (12) number of fecal boli, (13) locomotor activity (number of open and closed arm line crossings). From these results the percentage of entries into the open arms and closed arms based on the total arm entries were calculated for each animal. The percentage of time spent in the open arms and the closed arms was calculated over the 5-min test. The index of open arm avoidance (Trullas & Skolnick 1993) was calculated as 100 – (% time on open arms + % entries into the open arms)/2.

Open field

Mice were tested in the open field test chamber (72 × 72 cm square with 36 cm walls) for 5 min on each of 5 consecutive days. The floor was divided into 16 squares (18 × 18 cm) with painted lines; a separate square of equal size was drawn around the center of the box. Testing was performed in the dark phase under a 60-watt red light. Behaviors were videotaped and analyzed with the event-recorder program Hindsight v. 1.5 for MS-DOS. Mice were placed into one of the four corners of the open field, facing the center and tested for 5 min, after which the mice were returned to their home cages and the open field cleaned with 70% ethanol. The behaviors scored (Brown *et al*. 1999) included: (1) frequency line of crossing with all four paws, (2) frequency of crossing into the central square, (3) time spent in the central square, (4) frequency of free rearing, (5) frequency of rearing against a wall, (6) frequency of stretch attend postures, (7) frequency of grooming, (8) freezing, (9) number of puddles or streaks of urine, (10) number of fecal boli. Each animal was given a total locomotor activity score calculated as the sum of line crossings and rearing frequencies.

Novel object task

The novel object recognition task (Podhorna & Brown 2002) was conducted in the open field on the day after the final open field test. During the first trial, two objects (01 and 02) were placed in diagonally opposite corners of the arena, each 10 cm from the corner. Each mouse was placed into one of the four corners of the open field and tested for 5 min. The following behaviors were recorded: the number of line crossings, rearing and rearing against the wall, the frequency of approaches to each object and the time sniffing each object. The novel object duration ratio was calculated as the time spent with the novel object divided by the time spent with both objects on day 2. The mice were returned to their home cage for 30 min and then reintroduced into the arena for trial 2 with the familiar object (01) in the same position as in trial 1 and a new object (N) that replaced 02. The same behaviors as described above were recorded for 5 min. Metal objects of various shapes (jar lids, bolts, nuts) ranging in size from about 2 × 2 × 2 cm to 2 × 4 × 6 cm were used as objects. Object 2 (to be replaced in trial 2) was counterbalanced for each group of animals. Object 1 (to remain for trial 2) was the same for all mice, to eliminate the possibility of aversion or preference to the object. The new object was similar in size to 02 to reduce preference for either object. All objects and the apparatus were cleaned after each test using 70% ethanol to eliminate olfactory stimuli.

Olfactory learning and memory tasks

Working memory odor recall digging tasks were based upon those previously described for rats (Fortin *et al*. 2002), as modified by Katz *et al*. (2003). In brief, F1 hybrid ('HYB') and FVB mice in separate experiments were tested after 3 months of age following reduction to a constant weight of approximately 28 g on a fixed calorie diet of 8.5–10.2 kilocalories per day (five to six 500 mg pellets; #F0171, BioServ, Frenchtown, NJ). A sliver of increasingly buried cereal was then used to shape the mice to dig in 1.4 cm high × 4.5 cm diameter cups containing aquarium gravel scented with various spices ('odor cups'). Testing took place in the home cage into which were placed trays holding one or three odor cups. Each trial of the olfactory '2-sequence' task had two phases: first, the mice were exposed sequentially to two cups with different odors (presentation phase); then, after a delay of 15 seconds to 30 min, the mice were exposed simultaneously to the two presentation odor cups as well as a third, novel odor cup (choice phase). The mice were given positive reinforcement of small cereal food rewards by drop-in for digging in the presentation cups in the correct order, and mild tail pinch with padded tongs for digging in the wrong cups. Twelve trials per day were performed for 15 days. Each trial used a unique sequence of three odorants from among 25 available; these were shuffled daily. Percentage of trials in which mice chose odor 1 then odor 2 (perfect performance), odor 1 then 3, odor 2 first or odor 3 first were tabulated. Each trial was also given a score in which three points were given for choosing odor 1 then 2, two points for odor 1 then 3, one point for odor 2 first and zero points for odor 3 first.

Barnes maze

The Barnes maze (Barnes 1979) for mice is a circular platform 69 cm in diameter with 16 equally spaced escape holes around its perimeter and surrounded by a 15 cm high wall (Pompl *et al*. 1999). One of the holes allowed escape from the platform into a box. The platform was bathed in bright light (2580 lux) and had a buzzer above the center. Three visual cues (two geometric shapes and a picture) were taped to the inside of the wall, semi-randomly, in locations that were not directly above any of the escape holes. The test procedure had four phases: habituation (1 day); acquisition training (4 days); reversal training (4 days); and a probe trial (1 day).

On the first day, the mice were habituated to the maze and to their escape hole. Mice were randomly assigned one of four escape holes (numbered 4, 8, 12 or 16). An inverted 2-1 glass beaker was placed over the mouse and around the escape hole for 5 min. The beaker prevented the mouse from moving away from the hole, but allowed it to see the rest of the maze and to explore and enter the escape hole to avoid the bright lights of the maze. The buzzer was not used during this habituation trial. In acquisition training the mouse was released into the center of the maze and the latency to enter the escape hole with all four feet was recorded, as were the number of errors committed (head dips into incorrect holes) before the mouse located the escape hole. Repeated head dips into the same hole, without the animal moving away from the hole between dips, were counted as one error. Nose pokes into the correct escape hole without entry were not counted as an error. If the mouse did not enter the escape hole within 30 seconds, the buzzer was turned on to increase the aversiveness of the maze. The buzzer remained on until the mouse entered the escape box. If the mouse did not enter the escape box within 5 min it was guided to the escape hole with a piece of cardboard. Mice were allowed to remain in the escape box for 30 seconds before being returned to their home cages. The maze was cleaned with 70% ethanol between animals. Each mouse received four trials per day with an intertrial interval of about 20 min. Acquisition training continued for four days, for a total of 16 trials. During reversal training, the escape box was moved to the hole opposite that used during acquisition and mice were then tested using the same procedure as during acquisition training. A five minute probe trial with no escape box or buzzer was conducted on day 10. The maze was divided into four pie-shaped quadrants labeled 'correct' (the correct hole during reversal training), 'opposite' (the correct hole during acquisition training), 'right' (to the right of the correct quadrant) and 'left' (to the left of the correct quadrant). The following measures were recorded: (1) frequency of entry into each of the four quadrants, (2) duration of time spent in each of the four quadrants, (3) frequency of freezing, (4) duration of time spent freezing, (5) number of head dips in the correct and opposite holes.

Morris water maze

A MWM modified for mice was used (Paylor *et al*. 1996). Several wall posters, as well as furniture, acted as extra-maze visual cues. During testing, the room was dimly lit with diffuse white light. Mice were tested in four phases: acquisition training with a hidden platform; reversal training with a hidden platform; a single probe trial with no escape platform; four trials with a visible platform.

All mice were randomly assigned a start position (West, North, East or South) and each trial lasted a maximum of 60 seconds. Latency to climb onto the platform was recorded. If the animal did not find the platform within 60 seconds, it was guided onto the platform. Mice had to remain on the platform for 20 seconds before being removed. Each mouse completed four trials per day for three days of acquisition training. Then the platform was moved to the opposite quadrant and four trials of reversal testing commenced for three days. The interval between trials for each mouse was 10 min. On the probe trial (day seven), the following behaviors were measured: (1) frequency of entry into each quadrant, (2) duration of time spent in each quadrant, (3) number of times the mouse crossed the location of the platform during acquisition and reversal training (annulus crossing), (4) frequency and duration of thigmotaxic behavior, defined as swimming near the wall in the outer 1/6 of the radius of the pool.

8-Arm radial maze

The apparatus and protocol used for the spatial working memory task in the 8-arm radial maze was based on the win-shift paradigm described previously (Crusio *et al*. 1993; Schwegler *et al*. 1990). Eight extra-maze cues were positioned on the platform between the arms of the maze in a fixed configuration. Five days prior to training the mice were placed on a food restriction schedule to maintain them at 80–85% of their free feeding weight. On days five and six the mice received two 10-minute habituation trials, 24 h apart, with free access to all arms, with no food in any of the arms. The mice were trained for 15 consecutive days, one trial per day. For each animal a specific subset of four arms was randomly selected (with the provision that no more than two arms were beside each other) and a food reward was placed in the dish in each of the baited arms. An arm entrance was scored if an animal entered an arm with all four paws. Trials were terminated after the mouse entered all four of the correct (baited) arms.

Two configurations of the 8-arm radial maze were used. In the first (radial maze I), the center platform was surrounded with a clear Plexiglas barrel (20.4 cm in diameter, 31 cm high) which had 4×6 cm entrances into each arm. Within this barrel was a second removable clear Plexiglas barrel (18.5 cm in diameter, 33.5 cm high) with a guillotine door. At the start of tests using the concentric barrel apparatus, animals were given access to all arms. Once the mouse

entered the chosen arm, the inner barrel was lowered and the sliding guillotine door positioned in line with the arm, allowing escape back into the center platform. Once the mouse entered the center, the guillotine door was closed, preventing the mouse from entering any other arms for 5 seconds. In the second configuration (radial maze II), the entrance to each arm had a clear plastic guillotine door. For a door to be opened, the mouse had to touch or rear up against it for at least 3 seconds ('strong choice'). In the concentric barrel apparatus (radial maze I), if a mouse entered a baited arm but did not eat the food pellets, it was still recorded as an entry into the correct arm. Thus the mouse did not need to re-enter the correct arm to eat the food for this arm entry to be recorded. In contrast, in the individual door apparatus for which a definitive signal from the animal was required for a door to open (radial maze II), a correct score was counted only when the animal ate the food at the end of the arm. Entry into a correct arm without eating the reward was counted as a motivation error.

Measures recorded: (1) the sequence of arms entered, (2) the total number of arms entered until the last of the four baited arms had been entered, (3) the number of correct entries in the first four entries, (4) working memory errors (the number of times the mouse entered a correct arm after the initial entry), (5) reference memory errors (the number of incorrect, unbaited arms entered), (6) total time taken until all four baited arms were entered, (7) arm entry angles (the angle between consecutive arm entries).

Startle response and pre-pulse inhibition

Wildtype and *fmr1* KO mice approximately 6 months old were tested in an SR-Laboratory startle reflex apparatus (San Diego Instruments, San Diego, CA) with background white noise adjusted to 65 dB. Ten minutes prior to the onset of the startle session, all mice were given a saline injection (1.0 ml/kg, i.p.) in order to establish testing parameters for subsequent drug studies not reported here. Mice were placed in the startle apparatus and given a five minute adaptation period, after which each mouse was presented with six startle stimuli (40 ms, 120 dB) to assess initial startle magnitude. Mice were then presented with five different trial types in pseudo-random order such that a particular trial type was not presented more than twice in a row. Trials were separated by a variable intertrial-interval of 15 seconds (range 5–25 seconds). The trial types were as follows: 10 additional startle trials (120 dB, 40 ms), 10 trials of a 68 dB pulse (40 ms) followed 100 ms later by the startle stimulus, 10 trials of an 80 dB pulse (40 ms) followed 100 ms later by the startle stimulus, five trials of the 68 dB 'pre-pulse' (no startle stimulus was presented on this trial) and five trials of the 80 dB pre-pulse only (again, no startle stimulus was presented). At the end of the session mice were presented with five additional trials of the startle stimulus to assess habituation to the startle stimulus. Mice were tested weekly for three weeks to assess any changes in response over time and over repeated testing. Due to an equipment failure, data for six mice in each group were not recorded on the initial test day. Pre-pulse inhibition (PPI) of the startle response was calculated as a percentage by the following formula: $PPI = 100 \times [(a-b)/(a)]$ where 'a' is the mean value of the 10 startle response trials in the middle of the session and 'b' is the mean startle response to the 10 trials in which the startle stimulus was preceded by a pre-pulse. This measure was calculated independently for each level of the pre-pulse (68 and 80 dB).

Audiogenic seizure susceptibility

Population audiogenic seizure (AGS) sensitivity was determined by exposing mice to a high intensity siren of frequency peak 1800–6300 Hz at an average sound pressure level of 125 dB at 11 cm (Personal Alarm, Model 49–417, Tandy Corporation) for up to 16 min in an empty plastic box with a sound-absorbent tile lid under which the siren was mounted. C57 and hybrid strains were found to have elevated seizure frequencies at earlier ages than the FVB strains, so the ages of AGS testing were adjusted as follows: FVB strains (WT and KO) were tested at 30 days while C57 and HYB strains were tested at 21 days (Q.J. Yan, M. Rammal, M. Tranfaglia, R.P. Bauchwitz, unpublished data). Data presented in Fig. 8 are for the following numbers of unprimed mice: FVB (11), FVB KO (20), C57 (13), C57 KO (6), HYB (33), HYB KO (8). Seizures were also scored by the time of occurrence (latency) during a 16-min test and by type: clonic, tonic, tonic-clonic and status epilepticus, which in these studies almost always led to death following a prolonged tonic seizure. Wild running and jumping almost always preceded the onset of seizures, and these were noted but not included in the number of seizures.

Statistical analysis

All cognitive and behavioral tests were conducted by experimenters blind to the genotype of the mice. Statistical analyses of parametric data were conducted by the appropriate *t*-test (after first assessing relation between the variances) or by ANOVA. When ANOVA suggested a statistically significant difference, the Tukey-Kramer method was used to assess which differences in means contributed significantly. For non-parametric categorical data (olfactory learning and AGS) chi-square analysis and Fisher's exact test were used. Probability values $P < 0.05$ are indicated with an *, $P < 0.01$ with **, and $P < 0.001$ with *** in the figures. Error bars represent one standard error of the mean (SEM), unless otherwise indicated.

Testis weights

Testes from males aged 90–99 days trimmed of epididymis and all associated fat were weighed individually on an Ohaus Explorer balance (Pine Brook, NJ) with four digit significance (to 1/10 mg). The following number of subjects were used: C57 (19), C57KO (11), FVB (28), FVBKO (17), HYB (16) and HYBKO (13).

Results

Nonspatial learning and memory

Novel object task

In the novel object recognition test, mice with better object recognition memory show a preference for a novel object, i.e. they explore the novel object more often and for a longer period of time, while at the same time habituating to and spending less time with an object common to both trials (Ennaceur & Delacour 1988). When a novel object test was performed with WT and KO littermates on a FVB/NJ × C57BL/6J ($F \times C$) hybrid F1 background, there was no statistically significant difference by genotype in time investigating the novel object (Fig. 1a). As expected, there was a significant decrease in exploration of the common object by both genotypes on day 2 (Fig. 1b). An analysis of the novel object duration ratios showed that there was no difference by genotype: $F_{1,22} = 0.274$, $P = 0.61$.

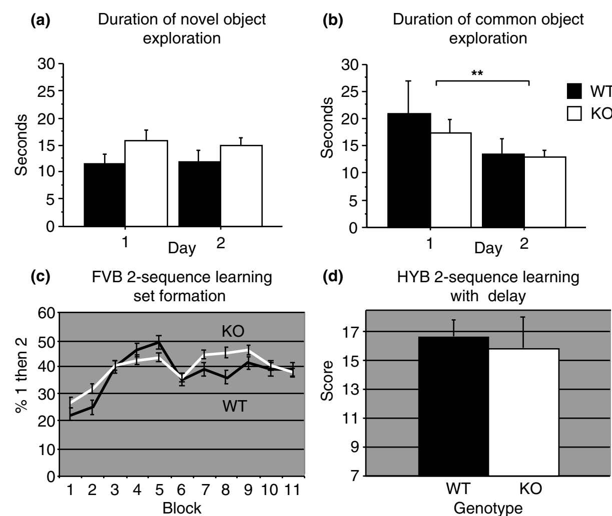

Figure 1: Visual and olfactory working memory comparisons. Visually-based working memory was tested using a novel object task in which animals were assessed for their ability to discriminate between a novel and a previously examined object after a 30-min delay. (a) Duration of novel object exploration. (b) Duration of common object exploration. There was a reduction in exploration of the common object between day one and two of the novel object test (**$P < 0.01$). Olfactory based learning set formation and working memory was assessed using a 2-sequence digging task in which animals had to remember the sequence in which two odorants had been presented to them after delays of 15 seconds or 3 min. (c) Rolling 3-day average for percent trials with perfect responses (choosing odor one followed by odor two) by FVB and FVB KO mice in the 2-sequence task with a 15 second delay (d) Plateau performance mean score by hybrid (HYB) WT and KO mice in the 2-sequence task after a 3-min delay. Perfect performance on the 2-sequence task would produce a score of 18 and random guessing a score of 7. There was no statistical difference between wildtype ('WT') and *fmr1-tm1Cgr* mutant ('KO') 2-sequence performance by *t*-test for FVB or HYB backgrounds.

Olfactory sequence working memory

An odor-based digging task was performed to specifically address whether a deficit existed in working memory for sequences of information, which has been implicated in the Fragile X syndrome (Dykens *et al.* 1987; Hodapp *et al.* 1992; Kemper *et al.* 1988). This task was also meant to assess the higher cognitive ability of the mice. The sets of three odorants used in each trial were novel and constantly shuffled, such that only by acquisition of a learning set, namely choosing odor one first and then odor two second, could the animal consistently achieve near errorless performance. FVB and FVB KO mice were able to improve their perfect sequence choices from near random (22% average) to a peak of 50% average (Fig. 1c). No statistically significant difference in performance between genotypes (WT and KO), either by day or for cumulative scores, was observed using either a 15 second or 3 min delay between exposure and challenge. Hybrid WT and KO mice also had no difference in plateau performance (Fig. 1d), but this background performed significantly better than the FVB and FVB KO (not shown; Katz *et al.* 2003). Thus, although a genetic effect was observed in this task, it was not associated with the *fmr1-tm1Cgr* allele.

Spatial learning and memory

Barnes maze

In order to further assess the cognitive performance of the *fmr1-tm1Cgr* ('KO') mice, several tests of spatial learning and memory were performed. During the first three days of training in the Barnes maze, WT mice made more errors (Fig. 2a) and had elevated latency to escape (Fig. 2b) compared to KO mice, although such differences did not rise to the level of statistical significance. Elevated anxiety may have had a negative effect on performance. Latency to the escape hole in this test increased by day (Fig. 2b), which would be expected if the maze was becoming less aversive to the mice with exposure. Although both genotypes entered all quadrants with equal frequency on the probe trial (no escape box; Fig. 2c), WT mice spent significantly more time in the correct quadrant than the KOs (Fig. 2d): X^2 (3,571) = 30.1, $P < 0.001$.

Morris water maze

In the MWM test, WT mice were slower to reach the platform on the first four days of testing, the first three of which were the acquisition phase (Fig. 3a). Although the difference in latency did not reach statistical significance: $F_{1,22} = 0.76$, $P = 0.39$, the overall shorter latency by the KOs in the acquisition phase is consistent with results in the Barnes maze. In the MWM probe trial, the WT and KO mice spent more time in the correct quadrant than in any other quadrant, with no significant difference by genotype (Fig. 3b). The similarity of WT and KO recollection of the platform site was

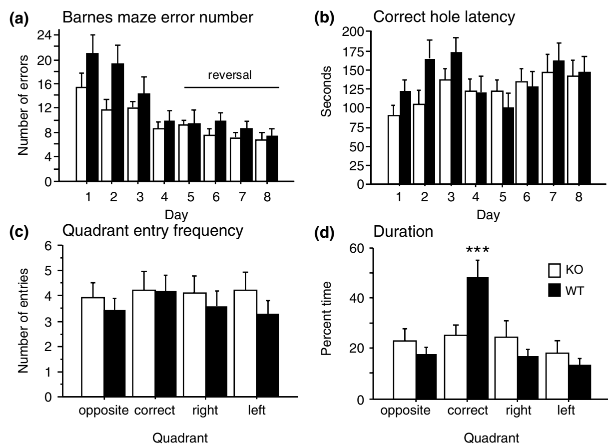

Figure 2: Barnes maze spatial memory test. (a) Number of errors prior to entering the correct escape hole. (b) Latency to enter the correct escape hole over eight days of training. (c) Frequency of correct quadrant entries during probe (no escape) test. (d) Time spent in each quadrant during the probe trial. *** $P < 0.001$.

more specifically measured in a circular region (annulus) around the last location that the platform occurred in the reversal trials; again, no difference between WT and KO performance was observed (Fig. 3c).

8-Arm radial maze

A radial maze test was performed and scored using a win-shift paradigm with four baited arms as previously described (Mineur *et al.* 2002; radial maze I). Over the first six days, the WT mice appeared to perform better in the maze than the KOs (Fig. 4a,b). ANOVA indicated a marginal difference between genotype means for working memory: $F_{1,19} = 4.6$, $P = 0.045$, but no significant differences were observed between any single pair of blocks by genotype using the Tukey-Kramer honestly significant difference test. ANOVA indicated a significant difference between genotypes in reference memory errors: $F_{1,19} = 9.2$, $P = 0.007$. A Tukey-Kramer *post hoc* comparison indicated that the difference in reference memory involved performance in the first three-day block (critical difference CD = 2.3; the mean difference on day one between genotypes was 2.6). The trend of these results was similar to results reported recently for *fmr1-tm1Cgr* mice which were interpreted as indicating a deficit in working memory (Mineur *et al.* 2002). However, when radial maze testing was extended in this study, it was observed that *fmr1-tm1Cgr* mouse performance became equal to that of their WT littermate brothers by the third block of trials (Fig. 4a,b). Therefore, it appeared unlikely that a memory deficit existed in the KOs as this would no longer apply at later testing times. Instead, it might have been that the rate of learning of the rules of the test differed between the genotypes, or alternatively, a difference in attention or

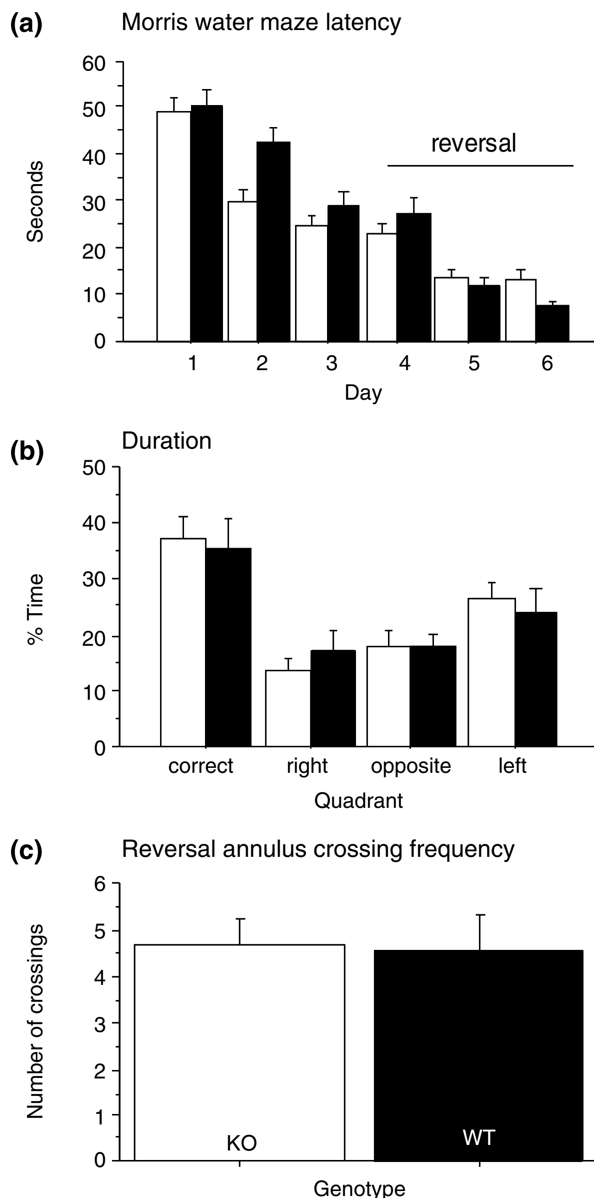

Figure 3: Morris water maze. (a) Mean latency to reach a hidden platform in the initial position and a new position ('reversal'). (b) Duration in correct quadrant during probe trial (no platform). (c) Frequency of crossing into a fixed zone around the previous reversal platform position during a probe trail.

search strategies existed. It was apparent that some mice were attempting to use a serial search strategy in the early days of testing, which might have affected the early performance if one genotype had differences in such tendencies. Keeping all arm doors blocked for a short time (usually about 5 seconds) when the mouse was in the center platform was intended to disrupt the effect of serial search strategies (Bolhuis *et al.* 1986) and did so in our tests. There was no

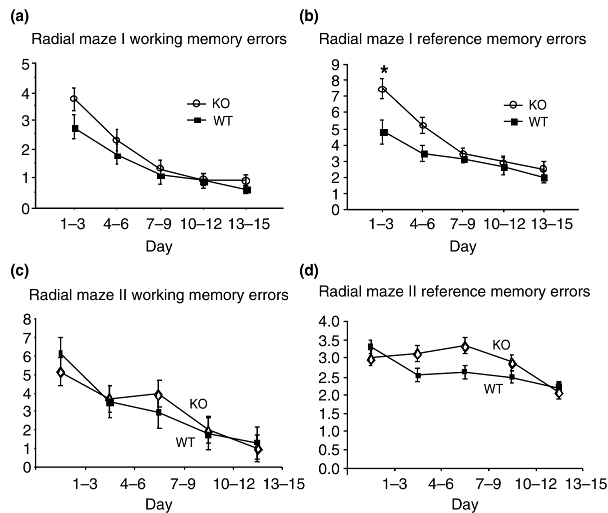

Figure 4: 8-Arm radial maze. Radial maze I: (a) Three day mean working memory errors (re-entries into a baited arm). (b) Three day mean reference memory errors (the number of entries into unbaited arms). Radial maze II: a second 8-arm radial maze performed with a second group of WT and KO FVB/NJ × C57BL/6J hybrid (F1 generation) littermates. Radial maze II had individually controlled doors to each arm which were opened when the mouse touched or leaned against the door for at least 3 seconds ('strong choice'). (c) Mean working memory errors (baited arm re-entries). (d) Mean reference memory errors (empty arm entries). In both tests, trials were continued until all four rewards were eaten.

statistical difference in the angles chosen between consecutive arm entries between the two genotypes (measured in groups of 3 days for 1, 45, 90, 135 and 180 degrees). However, in opening the doors after a fixed time, we observed that many mice on early days would enter the arm they happened to be nearest when the doors were unblocked, even though they had been moving to many arms (often serially) while the doors were blocked.

In order to further examine whether there was a reproducible difference between WT and KO animals during the first week of training, a second group of hybrid WT and KO littermates was tested (radial maze II). However, in the second maze animals were trained that opening a door required touching or leaning against it for at least three seconds. Thus, the animal learned to make a clear choice in order to get a door to open ('strong choice' test). If the animal were remembering the location of the food early in the testing, then it would not be expected that such a more definitive assessment of choice would have any negative impact on performance. In addition to this modification, the scoring was adjusted such that if an animal entered a correct arm but did not eat the reward, a motivation error was noted; when the animal did subsequently enter the arm and eat the food, it was scored as a correct response. However, the frequency of motivational errors was extremely small (6 such errors for all 16 mice over all days, of which 1 occurred on the first day, and the rest on the last day, four from a single animal), so this had no appreciable effect on the outcome. In this second radial maze experiment, there was no statistically significant difference in working memory errors (arm reentries) on any day by genotype (Fig. 4c). Only during one three-day block (days 7–9) did KO reference memory errors (empty arm entries) appear to be slightly greater than WT (Fig. 4d; $P = 0.13$), but this was due to performance of a small subset of animals and was not sustained throughout the test.

In comparing the two radial maze results, mice of both genotypes, but especially the KOs, made more reference memory errors (empty lane entries) in radial maze I than in radial maze II (strong choice). Conversely, both genotypes made fewer early working memory (reentry) errors in radial maze I than II. Although final performance was similar in the two maze formats, it took several days longer for the mice of both genotypes to master the maze (II) that required a definitive choice by the mouse.

Hyperarousal and anxiety

Open field

In the open field test, KOs entered the center more often, $F_{1,22} = 4.2$, $P = 0.05$, and spent significantly more time in the center, $F_{1,22} = 5.2$, $P = 0.03$, than WT littermates on each of 5 days of testing (Fig. 5a,b). No mean difference in genotype behavior by day rose to statistical significance using Tukey-Kramer *post hoc* analysis. The preference of KO animals to enter the center of an arena well before WT littermates, particularly on initial exposure, was quite evident in other tasks such as a Y-shaped water maze (data not shown). However, the KO animals also showed more stretch attend postures, suggestive of risk assessment, than did WTs on days 1, 2 and 4 (WT stretch attend was greater than KO on day 3). Rearing and freezing activity did not differ between the strains (except for elevated WT freezing on day 3 – the same day they exhibited more stretch attend behavior). At the very least, the stretch attends do not support a simple interpretation that the WTs were more anxious than the KOs. Rather, differences in preference for moving to the center of the maze might suggest more aggressive exploratory behavior in the KOs. There was no difference by genotype in overall locomotor activity as measured by line crosses (Fig. 5c).

Elevated plus maze

The EPM is regarded as a robust measure of rodent anxiety (Handley & Mithani 1984; Lister 1987; Pellow & File 1986; Trullas & Skolnick 1993) which should be able to provide support for the open field results suggesting reduced anxiety in KO mice (Peier *et al.* 2000 and herein). However, in the EPM there was no significant difference between WT and KO in the number of open arm entries (the mean was actually less for KOs), closed arm entries, open or closed arm duration, entries and time in the central square, head dips over the side of an open arm, stretching or rearing. Figure 6

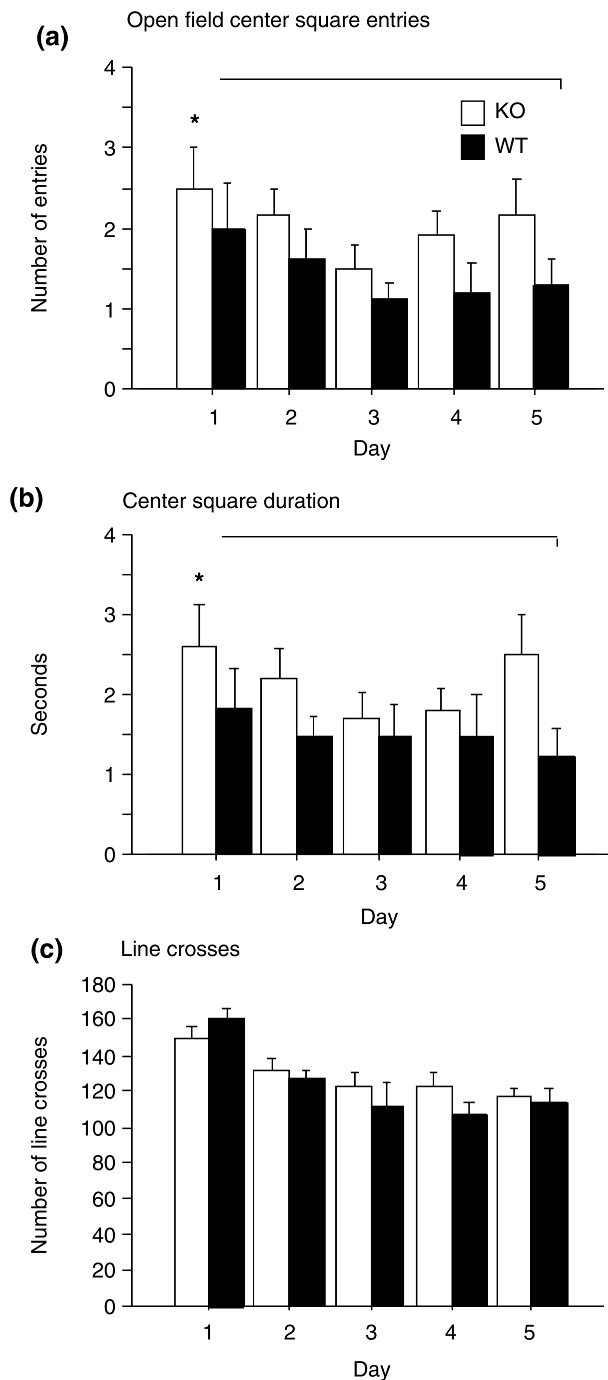

Figure 5: Open field. (a) Frequency of center square entries. (b) Duration in the center square. (c) Number of line crosses as a measure of locomotor activity. No statistically significant difference by genotype. Open square entries ($P = 0.05$) and duration ($P = 0.03$) were significantly greater for *fmr1-tm1Cgr* KO mice across days by ANOVA. $*P < 0.01$.

summarizes the EPM results as an index of open arm avoidance (Trullas & Skolnick 1993). An additional 10 F1 hybrid mice of each genotype tested in an EPM with a dark-sided arm under bright lights confirmed the lack of difference between the genotypes in open arm avoidance (M. Rammal and R.P. Bauchwitz, unpublished).

Acoustic startle: habituation and pre-pulse inhibition

No differences were observed between *fmr1* mutant mice and their WT counterparts on any of the startle related measures (see Table 1). Analysis of the data for the subset of animals that were successfully recorded over all three days did not reveal any significant effects of genotype on the initial day of testing nor any interactions between day and genotype on any of the recorded measures. Because these data did not reveal any effects, the data were collapsed across all available days of testing to create an average score for each animal for each measure. Both groups had similar degrees of response to the initial startle stimulus ($t_{22} = 0.146$, $P = 0.885$). While a significant reduction in startle amplitude was observed over the course of the session (Fig. 7a) for both groups of mice ($F_{3,66} = 70.402$, $P < 0.001$) there were no differences between the WT and KOs on the rate of habituation ($F_{3,66} = 0.644$, $P < 0.589$).

The percent inhibition of the startle response was calculated for each of the pre-pulse levels (68 and 80 dB) and is shown for each genotype in Fig. 7(b). The 80 dB pre-pulse resulted in significantly more inhibition of the startle than the lower 68 dB pulse ($F_{1,22} = 553.4$, $P < 0.001$). However, there was no effect of genotype nor an interaction between these two factors (both Ps > 0.1). At least one previously published paper reported that F1 *fmr1-tm1Cgr* mice exhibited a startle response to the pre-pulse stimulus itself (Nielsen *et al.* 2002). However, in the current study neither the KO nor

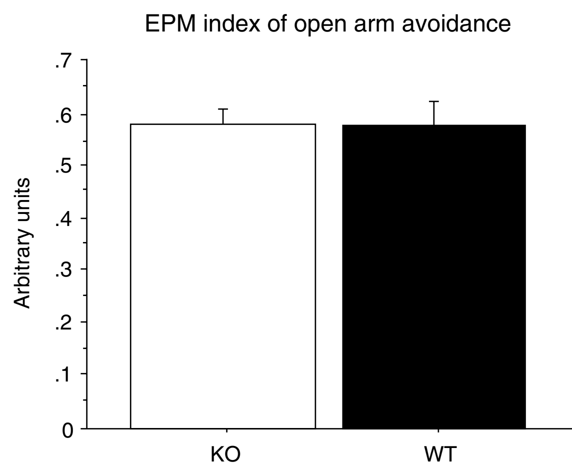

Figure 6: Elevated plus maze. Index of open arm avoidance by genotype measured as: 100 – (% time on open arms + % entries into open arms)/2. No significant difference by *t*-test.

Table 1: Startle response of F1 hybrid wildtype and *fmr1-tm1Cgr* mice

	Initial Startle	% PPI (68 dB)	%PPI (80 dB)
Wildtype	126.4 ± 14.2	32.7 ± 2.7	50.4 ± 3.5
Knockout	124.0 ± 8.2	37.4 ± 2.2	55.9 ± 2.1

Mean (± SEM) startle amplitude (in arbitrary units) during the initial startle trial and startle trials preceded by a prepulse of 68 or 80 dB.

WT mice showed any tendency to exhibit a startle response to the 68 or 80 dB signal when this signal was presented without being followed by a startle pulse ($Ps > 0.05$).

Audiogenic seizures

It has been reported that Fragile X KO (*fmr1-tm1Cgr*) mice are more sensitive to seizures induced by auditory stimulus than WT animals (Chen & Toth 2001; Musumeci *et al.* 2000). We found this to be the case for all strains tested: inbred FVB KO and C57 KO, and also F1 hybrid KO mice (Fig. 8). The *fmr1* mutation significantly raised the seizure rate in all WT backgrounds. In general, the FVB WT background was the most sensitive to audiogenic seizures of the WT strains. C57 WT were quite resistant to AGS under our conditions, yet the presence of the *fmr1-tm1Cgr* allele on this background substantially raised seizure sensitivity in total, and for status epilepticus (Fig. 8). Hybrid KO mice, regardless of the sex of the parental knockout strain (FVB × C57) or (C57 × FVB), had a much reduced susceptibility to status epilepticus (prolonged seizures most often leading to death) compared to the parental inbred KO lines (Fig. 8). From the lack of status epilepticus observed in hybrid KO mice, we conclude that this genetic background is most resistant to AGS, consistent with inbred AGS sensitivity being due to recessive alleles. Nonetheless, using the same equipment with a priming protocol, we could see AGS in all WT backgrounds, as well as elevated AGS frequency and severity in the susceptible FVB and KO lines (Q.J. Yan, M. Tranfaglia and R.P. Bauchwitz, unpublished data).

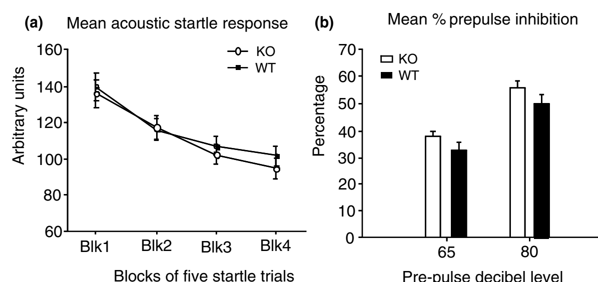

Figure 7: Pre-pulse inhibition of startle response in F1 hybrid WT and fmr1 mutant mice. (a). Mean acoustic startle response over blocks of five startle trials. (b) Pre-pulse inhibition of the startle response. In neither measure was there a statistically significant difference by genotype.

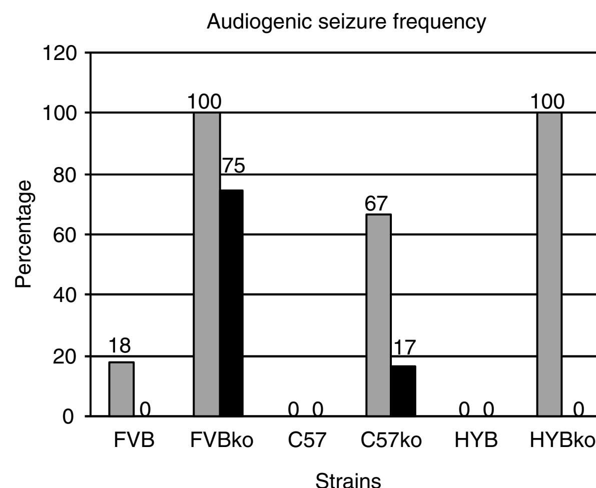

Figure 8: Seizure frequency in *fmr1-tm1Cgr* mice of various genetic backgrounds. FVB and FVB KO male mice at 30 days of age, and C57, C57KO, HYB and HYBKO at 21 days of age were exposed to high intensity sound from a siren. The lighter, left bar of each pair shows total seizures; the darker, right bar is status epilepticus. FVB is FVB/NJ, C57 is C57BL/6J, HYB is C57 × FVB, KO indicates the *fmr1-tm1Cgr* mutation.

Molecular characterization

It has been repeated many times in the literature that *fmr1-tm1Cgr* mice lack *Fmr1* RNA or protein (e.g. Brown *et al.* 2001; Li *et al.* 2002; Nielsen *et al.* 2002) or are 'null' (Miyashiro *et al.* 2003). However, upon performing RT-PCR of mRNA preparations from *fmr1-tm1Cgr* mice, we observed that *Fmr1* mRNA was present both 3′ and 5′ of the disruption site in exon 5 (Fig. 9a; Bauchwitz 1998). Northern blot analysis showed that the *fmr1-tm1Cgr* promoter is producing *fmr1* mRNA at significant levels, as assessed by probes specific for the 3′UTR of mouse *Fmr1* (Fig. 9b). By sequencing multiexon cDNA products from several *fmr1-tm1Cgr* specific PCR primer pairs located throughout the *fmr1-tm1Cgr* sequence, we demonstrated that *Fmr1* sequence, and not *Fxr1* or *Fxr2*, was being expressed in *fmr1-tm1Cgr* male mice.

Primers spanning the PGKneo insertion site in exon 5 of the *fmr1-tm1Cgr* allele were used to obtain cDNAs for sequencing (Table 2). Sequence of the KO RT-PCR products were definitive regarding the ability of the *fmr1-tm1Cgr* allele to produce splice products through and around the disruption. Some of the splice products obtained are diagrammed in Fig. 9(c) (see also GenBank accession numbers AF170530, AF179463, AY544163 and sequences in Fig. 10). In general, various truncated FMRP products were predicted. However, of potentially greater significance, one of the sequenced products encoded an mRNA with an ORF which could produce a protein extending the entire length of FMRP. This full length ORF is actually the product of a double splice in which exon 4 is joined to 153 bases of reverse orientation Tn5

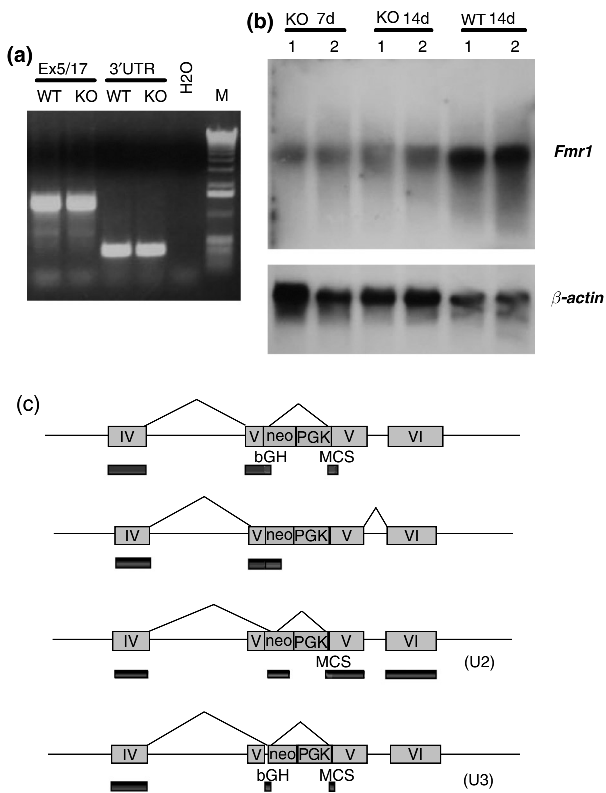

Figure 9: *Fmr1* mRNA is present in the fmr1-tm1Cgr mouse. (a) *Fmr1* RT-PCR shows common products in WT and KO mice in the 3′UTR, the 3′ coding region (spanning exons 5–17; 'Ex5/17'), and 5′ of the disruption site (exons 1–4; 'Ex1/4'). (b) Northern blot analysis of polyadenylated mRNA from hybrid KO and WT mouse brains (at 7 and 14 days of age). A strand specific RNA probe for the murine *Fmr1* 3′UTR shows significant levels of *Fmr1* mRNA in the *fmr1-tm1Cgr* mouse. The same blot was stripped and rehybridized with a probe for murine β-actin mRNA. (c) Schematic diagrams of some *fmr1-tm1Cgr* splice isoforms obtained from sequencing RT-PCR products. At least one splice product (bottom) can encode a protein which extends the full length of FMRP.

neomycin structural gene sequence, which in turn is joined to the multiple cloning site sequence found at the 3′ junction of PGKneo and exon 5 (Fig. 10a). Conserved splice acceptor and donor sequences (5′ intron GT and 3′ intron AG) were observed at the novel splice sites. The difference between the predicted protein and true FMRP is that 61 novel amino acids from non-coding neomycin (Tn5) strand and multiple cloning site sequence would replace 12 amino acids from the portion of exon 5 preceding the disruption. The sequence in Fig. 10(a) (as well as the others described in Fig. 9) rules out any possibility that the *fmr1-tm1Cgr* allele is not being expressed. These products were found in the brains of FVB, C57 and hybrid strains in the fetal, newborn, juvenile and adult periods. The same 3′ splice junction, spanned by the 3neoiso-L primer, was also present in conjunction with a 5′ splice between the KO *fmr1* exon 4 and the bovine growth hormone polyadenylation signal found 3′ of the neomycin insert sequence. This 3neoiso-U3 product was easily and widely observed in KO but not WT animals (Fig. 10b). As for the other PCR products, the identity of the 3neoiso-U3 products were confirmed by DNA sequencing (Fig. 10b).

More than one dozen additional splice isoforms were observed spanning the disruption site in the *fmr1-tm1Cgr* allele but not sequenced. Therefore, the products described here are not comprehensive and we do not rule out that other splice products with full length open reading frames may be present. The goal of this characterization was to demonstrate that *fmr1-tm1Cgr* is not a molecular null, i.e. it produces *Fmr1* mRNA, and possibly truncated or even full length FMRP.

In a preliminary effort to quantitate the level of the U2 splice product, RNase protection assays were performed. Knockout specific splice products were observed from the U2 region (Fig. 10a). The lower, darker product was of exactly the size (167 NT) predicted for a splice product lacking the 3′ junction of U2 (Fig. 10a). The upper product is almost exactly the size (185 NT) of the U2 full length open reading frame product (187 NT). The upper KO specific band was generally

Table 2: PCR primers for genotyping and molecular analysis of *fmr1-tm1Cgr* RNA

Name	5′	3′	Ta	cDNA
Neo-(N3/N4)	AGAGGCTATTCGGCTATGACTG	CCTGATCGACAAGACCGGCTTC	65	417
Ex5(5′)/5(3′)	ATAGAATATGCAGCATGT	TTGTCGTAAATCTTCTGG	50	138
MuSp 3′UTR	AGATGTTTTCAGCTACTTG	AAAAAAACCCCACAAAAAT	53	378
HuSp 3′UTR	TTGAATTTTCATTTTACAG	CCTTGGTTAATTATCTACA	53	238
Ex5/17	TCACAATTGAGCGTCTAC	ACGGAAATGGTATAGGAAATA	60	1571
Ex1/4	TCCAATGGCGCTTTCTAC	CCTTTATCATCCTCACTT	52	235
Ex4/5	GCCTTGCTGTTGGTGGTTAG	GCAGGTTTGTTGGGATTA	60	138/var
Ex3/7	GAGACAGATTCCATTCCA	TCGCTTTGAGGTGACTTC	60	424/var
3/5(3′)	GAGACAGATTCCATTCCA	TTGTCGTAAATCTTCTGG	60	301/var
3neoiso-U/L	ATTCCCACCACCTGTAGGTTA	GATAAGCTTGATATCGAATTCTTG	54	300

cDNA sizes are identical for WT and KO unless spanning the KO insertion site in exon 5, in which case 'var' for ' various sizes' is indicated. For KO full length isoform specific product (3neoiso primers), size is for KO only.

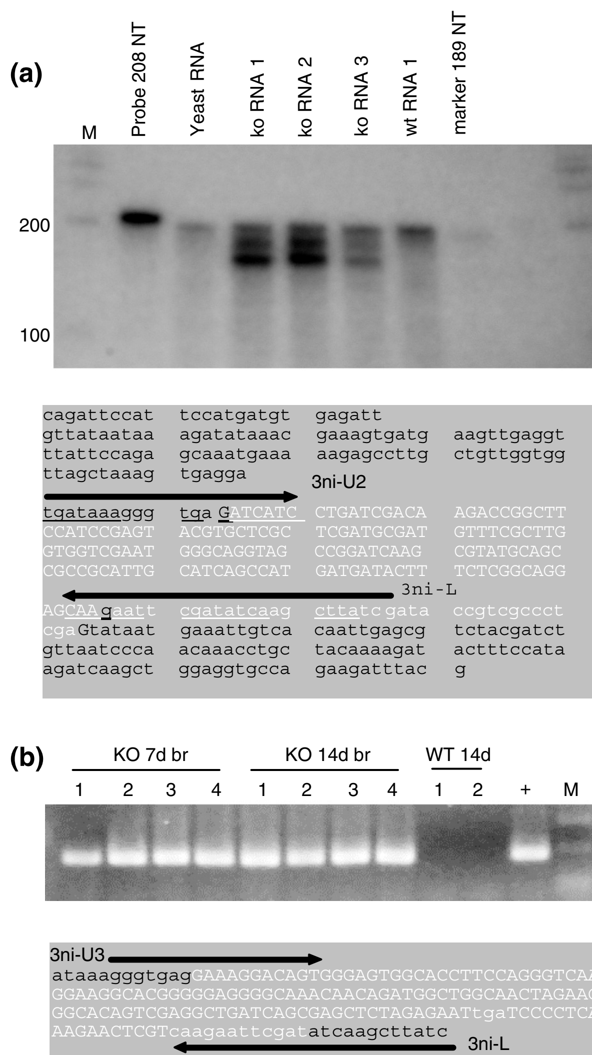

Figure 10: RNase protection, RT-PCR and sequence of *fmr1-tm1Cgr* isoforms. (a) Upper panel: RNase protection assay with 208 NT RNA probe spanning the U2 *fmr1-tm1Cgr* 5′- and 3′ splice junctions. Two *fmr1-tm1Cgr* allele specific products are observed: a lower band of 167 NT and an upper band of 185 nt. A third band of ~ 190 NT is common to KO, WT and yeast RNA samples. M = markers (in nucleotides = NT). Lower panel: exon 4 to exon 5 KO U2 splice sequence across the PGKneo insert sequence which allows production of a full length FMRP. The 153 base reverse strand neomycin sequence is in uppercase white and the 38 base polylinker sequence in lowercase white italics. Sequence from *fmr1-tm1Cgr* exon 4 and the 3′ end of exon 5 are in lowercase black. Splice points are indicated with bold lettering. The 3 neoiso-U2/L primer pair, indicated with arrows, crosses the exon 4-neo and neo-polylinker splice junctions. GenBank accession number is AF179463. (b) RT-PCR products and their DNA sequence from hybrid *fmr1-tm1Cgr* mice using knockout specific 3neoiso-U3/L primers. M = markers; the product is 178 bp in length.

found to be about 1/10th the level of the lower KO specific product. These two KO specific bands were present in all KO brain total RNA samples tested (5 FVB KO adult and 3 HYB KO 7–15 day postnatal), but not in any WT (2 FVB adult and 1 HYB 7 day postnatal). The quantity of the two KO specific bands observed in the RNase protection assay was estimated by comparing signal intensities to a titration of known amount of target RNA (*Materials and methods*). The upper KO specific band was present at a level of approximately 2–4 pg and the lower 167NTband about 20 pg, although the relative levels of the upper band could appear to be greater in some samples (Fig. 10a). Since adult hemibrains of approximately 200 mg produced 240 μg total RNA on average, a 50 μg RNA sample was derived from 42 mg brain. Using a density of neurons in the mouse brain of 180 000/mm3 (Jerison 1973), and molecular weights of 60 300 and 50 000 for the 187 and 167NT products, respectively, it was estimated that there may be at least 100 copies of the 167 NT splice product and on the order of 10 molecules of U2 per murine neuron on average. The levels of the U2 KO RNAs might be very different if specific populations or developmental time points are examined. Efforts to quantitate expression levels during development and to obtain amino acid sequence information from *fmr1-tm1Cgr* specific protein products observed with antibodies to FMRP are underway.

Testis weights

It has been demonstrated that there exists a substantial increase in testicular size in *fmr1-tm1Cgr* strains relative to WT controls, with a maximum difference in adults at 3 months of age (Dutch–Belgian Fragile-X Consortium 1994; Kooy *et al.* 1996). This is consistent with the enlarged testicular volumes observed in males with FXS (Butler *et al.* 1992). In order to ensure that our *fmr1* KO mice were indeed what the molecular genotyping suggested, we measured testis weights for all strains used in this study (Fig. 11). In all three genetic backgrounds of WT and KO mice, there was a highly significant difference in weight between WT and KO testes. This supported the audiogenic seizure data indicating that the *fmr1-tm1Cgr* allele was penetrant in our strains. The testis data also indicated that strain background had a significant effect on adult testis size. Hybrid WT testes were approximately the same size or larger, on average, than the testes of the inbred KO strains (Fig. 11; HYB vs. C57 KO $P = 0.13$; HYB vs. FVB KO $P = 0.001$). It is known that testis size does not correlate with body size, but rather with chronological age (e.g. Zachmann *et al.* 1974). This suggests that some recessive alleles in the inbred strains were acting to reduce adult testis size relative to that seen in the hybrid animals. Furthermore, the effect of such alleles had a magnitude approximately equal to that of the *fmr1-tm1Cgr* allele itself (Fig. 11). This contrasts with the case of adult humans with FXS, in which the range of testicular size has little overlap with that of normals; the 50th percentile of mean

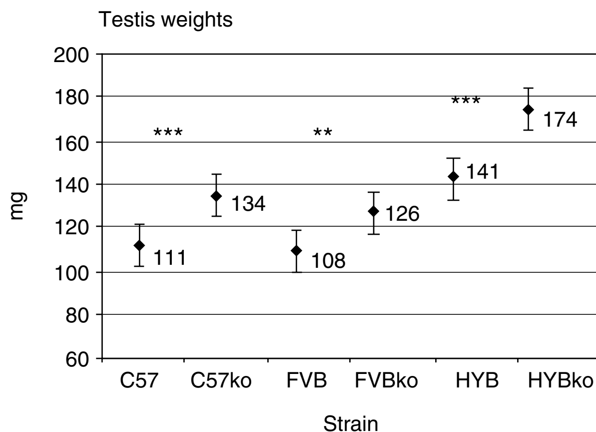

Figure 11: Testis weights for three murine genetic backgrounds with and without the fmr1-tm1Cgr allele. Testes from 90 to 99 day old animals (peak size) were trimmed and weighed. Highly significant differences were apparent in all WT and KO pairs within a genetic background (C57BL/6J, FVB/NJ, and F1 hybrid of C57 × FVB). $^{**}P < 0.01$, $^{***} P < 0.001$.

FXS testis volume is almost 100% larger than the 95th percentile of normal males (Butler *et al.* 1992). The average relative increase in KO testes weights for all strains measured here was 20.3% (Fig. 11), as compared to more than 150% for FXS males over the age of 17 years (Butler *et al.* 1992).

Discussion

It was our intent in these experiments to perform a survey of behavior of *fmr1-tm1Cgr* mice on an F1 hybrid genetic background, with an emphasis on working memory performance. Startle tests in *fmr1-tm1Cgr* mice were originally performed with our C57BL/6J × FVB/NJ ('CxF') F1 generation hybrid mice as proposed by one of us (RPB; Nielsen *et al.* 2002). Our rationale for use of such mice, created by crossing two inbred strains, was that the inbred strains themselves are known to carry a number of homozygous mutations which can substantially affect behavioral and cognitive testing. For example, C57BL/6J mice develop age related hearing loss (Johnson *et al.* 1997), while FVB/NJ mice carry the *rd1* (*Pde6brd1*/*Pde6brd1*) allele for retinal degeneration and are therefore essentially blind as adults (Pittler & Baehr 1991). Murine genetic background has also been shown to affect the pre-pulse inhibition of the startle response, with C57BL/6J showing less inhibition than FVB/NJ (Paylor & Crawley 1997). In animals with an F1 hybrid genetic background, by contrast, the effects of recessive alleles should be largely reduced or eliminated. Therefore, these behavioral studies should more closely define the effect of the *fmr1-tm1Cgr* allele itself in outbred *Mus musculus* populations, and would be predicted to be more comparable to effects in other outbred species, such as humans.

Learning and memory

Several tests of learning and memory were performed with the hybrid mice. The MWM, Barnes maze and 8-arm radial maze can measure ability to learn the position of visual-spatial cues over a period of days. The 8-arm radial maze, as well as novel object and olfactory sequence memory tasks can assess shorter term or working memory, which is known to correlate well with intelligence in humans. The olfactory tests were specifically performed in order to assess memory for sequences of information, a task which should be quite relevant to deficits observed in humans with FXS.

Morris water maze

The MWM has been repeatedly used to test *fmr1-tm1Cgr* KO mice (D'Hooge *et al.* 1997; Dutch–Belgian Fragile-X Consortium 1994; Kooy *et al.* 1996; Paradee *et al.* 1999), with mixed results. In the MWM, a rodent has to learn the position of a submerged, invisible platform in a pool, presumably using only distant fixed visual cues. The premise of the task is that a mouse or rat will be averse enough to floating or swimming in water that it will seek the most rapid means to exit by finding and perching upon a platform. Initial behavioral testing of the extant FX KO mouse (*fmr1-tm1Cgr*) on a C57BL/6J-129 mixed background showed that they had increased latency in the initial training trials and even more so upon the initial reversal trial (Dutch–Belgian Fragile-X Consortium 1994). Otherwise, however, the KOs learned at the same rate and achieved the same performance as the WT controls. There was no difference in the probe trials (in which the hidden platform was removed after its position had been learned). Absence of effect in a probe trial is important, since scoring of learning and memory may be more accurately reflected in this measure than in differences in latency to reach the platform (Gallagher *et al.* 1993; Hodges 1996). Further testing of MWM performance did not support the original difference in training trial latency between WT and KO animals (D'Hooge *et al.* 1997), but did substantiate differences in reversal trials. Reversal results in animal tests may reflect mental flexibility (or conversely, tendency toward perseveration) and require cortical function, particularly of the frontal lobes (Divac 1972; Kolb *et al.* 1974). Reversal results are of particular interest in humans with FXS since related measures (e.g. elevated perseveration in the Wisconsin Card Sorting Test) have been shown to be affected (Cornish *et al.* 2001; Wilding *et al.* 2002). However, it was also demonstrated that even when the platform was visible, KO mice reached it significantly more slowly than WT littermates, suggestive of some motor or performance difference between the genotypes which might complicate interpretation of the results. In addition, there was no difference in probe trials between WT and KO for acquisition or reversal, suggesting that both remembered the general location of where the platform had been (before removal for the probe trial). It was concluded that the MWM results did

not support significant involvement of the hippocampus in the *fmr1* mutant allele's effect (D'Hooge *et al*. 1997). In yet further MWM tests, *fmr1-tm1Cgr* animals on a C57BL/6J background with more than 15 backcrosses showed no deficits in the MWM reversals compared to controls (both latency and probe), while animals of a C57BL/6J × 129RE/J F1 hybrid background were found to have elevated latency for the training blocks occurring on the first day compared to controls (Paradee *et al*. 1999). Indeed, it was confirmed that normal 129/ReJ animals have a significantly increased swim latency compared to C57, so that such differences in latency observed previously may have been due to 129 alleles segregating with the *fmr1-tm1Cgr* mutation but removed during backcrossing to C57. A further study of *fmr1-tm1Cgr* on the C57 background again showed no difference in escape latency in the MWM (Peier *et al*. 2000). The results presented here, which used F1 hybrids of FVB/NJ and C57BL/6J, further substantiate the likely negative effect of 129 alleles since no deficit was observed in the presence of FVB alleles. Therefore, it can be concluded that *fmr1-tm1Cgr* on highly backcrossed C57BL/6J or F1 hybrid backgrounds that exclude most 129/ReJ alleles do not show deficits in standard MWM measures.

Barnes maze

In the MWM tests presented in this study, hybrid KO mice performed at least as well as if not better than their WT littermates, including in the reversal phase. This would tend to suggest that the KO allele itself does not promote a substantial deficit in performance of a standard MWM task, and by extension, the forms of visually based learning and memory examined. In order to further assess the meaning of the MWM spatial memory results, a Barnes maze was employed. The Barnes maze is a land-based circular maze in which mice are motivated to escape into holes located around the perimeter upon exposure to bright lights and noise. One advantage of the Barnes maze in testing mice is that they are generally less aquatic in habitat than rats, so their performance tends to be better in the land-based Barnes maze than in the presumably more stressful water mazes. In these experiments, we found that the KO animals acquired the task more quickly than WT littermates. The difference in performance was present from the very start of the testing (and then disappeared), as was the case for latency in the MWM. The results of Barnes maze probe trials in which the escape box was not present showed that the KO and WT entered the correct quadrant with the same frequency, but the WT animals stayed there significantly longer. This might mean that the WT had a more accurate memory of the location of the escape hole, or it might mean that the KO more quickly determined that no escape was present and left. Alternatively, WT mice may have had more apprehension that the buzzer would sound than the KO mice. (No buzzer was employed in the probe trial, but its use was required to get the mice to enter the escape box.) In contrast to the absence of aversive stimulus in the Barnes maze probe trial, the aversive stimulus in the MWM probe trial (water) could not be removed. Given that no probe trial differences have ever been observed between WT and KO in the MWM, it is not possible to conclude without further investigation what the difference in probe data in the Barnes actually mean. In particular, a difference in search strategy between the strains (spatial vs. sequential search) may be an important factor (R. Brown, unpublished data; see also Wolfer *et al*. 1998 for MWM search strategies).

8-Arm radial maze

A more stringent test of spatially-based memory was desired to assess whether milder deficits in the KO mice might be revealed. An 8-arm radial maze was used in two tests with two different groups of hybrid animals. In one test, scored as previously described for the KO animals (Mineur *et al*. 2002), we initially observed, as Mineur *et al*. did, that the KO animals appeared to acquire the task more slowly during the first week. However, we found that the performance of the KO became equivalent to the WT during the second week. If working memory is really measured by arm re-entry, as is assumed in this task, then we should conclude from such studies that the KO animals' working memory improved over time. As this is very unlikely to be the case, it seems possible that such re-entries might reflect something other than working memory, e.g. exploratory behavior, motivation for or comprehension of the task or a combination of such factors. Elevated reference memory errors (entry into arms never baited) by KO mice led to a significant difference in the first three days on radial maze I. Again, an elevation in KO exploratory drive early in the testing could explain the results. Alternatively, the KO animals may have been slightly slower to learn not to enter non-productive (unbaited) arms, possibly due to a slower formation of long-term memory during the first days of testing or less facility in comprehending the relationship between what was in the environment and the position of the baited arms.

The radial maze is known to be very sensitive to response patterns, i.e. search strategy preferences by the rodent (Olton 1987). Confining an animal to the central arena after it leaves an arm is meant to disrupt such patterns. We wished to further reduce residual patterns as well as any willingness of the animal to guess early in the testing. Therefore, we modified the radial maze protocol such that the mice had to very clearly indicate which of eight individual doors they wanted opened. In a subsequent test with this modified procedure (termed here 'strong choice'), we did not observe any significant difference between performance of the KO and WT mice. Therefore, in the absence of corroborating data from other spatial memory tasks, as well as from a modified procedure of the same task, we cannot conclude with certainty that any difference in early radial maze performance represents a difference in learning capacity.

Olfactory sequence working memory

Working memory in humans refers to the short-term, active maintenance of information allowing performance of complex tasks (Baddeley 1998). Working memory has 'executive' attention, as well as subservient information storage components. Working memory may also be more highly correlated with IQ than long-term memory (e.g. Numminen *et al.* 2000). Recent studies have confirmed deficits in FXS working memory (Munir *et al.* 2000a), including performance in a two-back test of working memory in which the subject must hold an image in his memory after viewing an intervening image and then perform a match to sample (Menon *et al.* 2000). In particular, it has been demonstrated that humans with FXS show impairment in sequential working memory, such as imitating a sequence of hand movements (Dykens *et al.* 1987; Hodapp *et al.* 1992; Kemper *et al.* 1988). Tests of working memory in animals often employ changes in visual-spatial information as can be presented in a radial maze (above). Alternatively, non-spatial, sequential working (or episodic-like) memory can be assessed using olfaction (Fortin *et al.* 2002). In this study, no difference was observed between WT and KO mice in acquisition or memory for two odors presented in sequence and then probed in the presence of an additional novel decoy odor. This was true even after a delay of 3 min between odor exposure and testing. Therefore, odor-based working memory does not appear to be grossly affected in *fmr1-tm1Cgr* mice, consistent with lack of difference in a spatially based working memory plus water maze test in these mice (Van Dam *et al.* 2000). The olfactory '2-sequence' digging task was also meant to assess higher cognitive functions such as those requiring rule based learning (see details in Katz *et al.* 2003). Again, no effect of the *fmr1-tm1Cgr* was detected, despite easily finding differences by strain background, suggesting that the effect of the *fmr1-tm1Cgr* mutation must be quite small or non-existent. It was also found in this study that visual memory for novel objects was not affected by the KO mutation. More stringent tests of working memory in these animals are underway; however, the results to date do not demonstrate signs of a large deficit.

Hyperarousal and anxiety

Aside from intelligence, another area of interest in FXS is a constellation of symptoms which might be described as part of a hypersensitivity or sensory hyperarousal syndrome, e.g. avoidance behaviors such as gaze aversion and tactile defensiveness, and response to excess excitement such as hand flapping and hand biting (Hagerman 1996; Hagerman *et al.* 2002). Males with full mutations of *FMR1* can show hyper-reponsiveness and diminished habituation to auditory, tactile and other stimuli (Miller *et al.* 1999; Rojas *et al.* 2001). Such effects include elevated anxiety (Bregman *et al.* 1988; Dykens *et al.* 1989), an elevated startle response with a diminished inhibition of that response by a prior stimulus (Miller *et al.* 1999) and a substantially elevated risk of seizures (Finelli *et al.* 1985).

Open field and elevated plus maze

Based on the high incidence of anxiety-related disorders seen in FXS, tests of the mouse model for this phenotype are worthwhile. Surprisingly, one study of *fmr1-tm1Cgr* mice found that in measures of spending time in the center of an open field and the number of transitions between light and dark chambers, these mice were seemingly less anxious than WT littermate controls (Peier *et al.* 2000), in contrast to what would be expected from humans with FXS.

In open field tests performed in this study, hybrid KO animals spent more time in the center of the field than their WT littermates, consistent with the previous results (Peier *et al.* 2000). However, subsequent testing in the EPM, another test which can demontrate anxiolytic sensitive behavior in rodents (Pellow & File 1986; Lister 1987; Trullas & Skolnick 1993), did not show any difference between the two genotypes (using the same group of animals, as well as an additional group of 20). It may be that time spent in the center of an open field did not represent a measure of reduced anxiety in these mice. An alternative explanation of the open field results might be that the KO animals have a different exploratory propensity than their WT brothers. Under a two factor theory, exploration and anxiety are independently competing motivators, such that anxiety may be constant yet exploratory drive different (Russell 1973). The factorial contributions of both emotionality and exploration on open field ambulation has been described (Ramos *et al.* 1997; Walsh & Cummins 1976). In addition, thigmotaxis during the first 5 min in the open field (the period employed here) is not very sensitive to drug effects on anxiety level (Simon *et al.* 1994), consistent with the idea that exploration need not be solely a function of anxiety level.

Some previous open field studies have suggested hyperactivity from observations of KOs (Dutch–Belgian Fragile-X Consortium 1994; Peier *et al.* 2000), while others have not (Nielsen *et al.* 2002). There was no significant evidence of hyperactivity based on line cross measures in the *fmr1-tm1Cgr* KO hybrid mice in this study (performed in low light which should enhance locomotion in this test, e.g. see Trullas & Skolnick 1993). Our data suggest that it is unlikely that notable hyperactivity in the open field up to 5 min is produced by the effect of the *fmr1-tm1Cgr* allele in adult mice in the absence of other genetic or environmental contributions.

Acoustic startle

The startle response involves sensory signaling through the brainstem which can be measured as physical movement of the eyelids or body (Koch 1999). The startle response can be inhibited, either by habituation (repeated presentation of the startle) or by presentation of a weaker stimulus approximately 100 ms prior to the strong startle stimulus (Braff *et al.* 2001; Geyer & Braff 1987). Inhibition of the startle (pre-pulse inhibition; PPI) is mediated via inhibitory signaling in pontine nuclei of the brainstem. There is evidence from

previously published data that *fmr1-tm1Cgr* KO mice can respond differently to auditory startle than WT animals. *Fmr1* KO mice can show a reduced response to auditory startle (Chen & Toth 2001; Nielsen *et al*. 2002) but this is not always observed (Peier *et al*. 2000; this study) and appears to depend on the amplitude of the startle stimulus (Nielsen *et al*. 2002).

Typically, animals will habituate to the startle stimulus, and it is interesting to note that this habituation response has been found to be deficient in neuropsychiatric conditions such as schizophrenia (Freedman *et al*. 1987; Geyer & Braff 1987) and sensory-modulation disruption (McIntosh *et al*. 1999). In a previous study of *fmr1-tm1Cgr* KO mice, C57 KOs demonstrated no habituation to a 120 dB startle stimulus, while F1 hybrid WT mice showed a trend towards disrupted habituation (Nielsen *et al*. 2002). In this study we found that there was no gross difference in habituation to the startle between the two genotypes in an F1 hybrid background; the downward sloping response curves are essentially parallel. Furthermore, in prior studies both C57 KO and FVB KO mice have been reported to have an enhanced PPI relative to comparable WT mice (Chen & Toth 2001; Nielsen *et al*. 2002). No significant difference in PPI of the startle response was observed in this study with F1 hybrid mice (C57 $\times$ FVB), consistent with recent data using an identical cross (Nielsen *et al*. 2002). These data support an interpretation that the enhanced PPI of the startle response reported for f*mr1-tm1Cgr* mice on a C57 background (Nielsen *et al*. 2002) may have been at least in part a strain-specific background effect, perhaps one related to hearing loss known to affect acoustic startle response in this strain (Carlson & Willott 1998; Willott & Turner 2000). The absence of baseline startle differences in the strains used here are consistent with the absence of difference in the EPM, since amplitude of the acoustic startle response and time spent in the closed arm are well correlated in the mouse (Trullas & Skolnick 1993).

Audiogenic seizures

Finally, it has been reported that *fmr1-tm1Cgr* mice have an enhanced susceptibility to AGS in the FVB/NJ and mixed FVB/129 backgrounds (Chen & Toth 2001; Musumeci *et al*. 2000). We confirm and extend those findings here. FVB KO mice had the most elevated susceptibility, with more than 80% of mice between 20 and 30 days of age dying from status epilepticus (a prolonged tonic seizure leading to respiratory arrest) within 1 minute under our conditions. C57BL/6J KOs are also quite sensitive, but hybrid KO animals are considerably less sensitive, as indicated by the lack of status epilepticus in that genetic background. Hybrid WT mice are completely resistant to AGS here, consistent with the AGS sensitivity of FVB and C57 inbred lines being due to recessive alleles. Therefore, our data suggest that the hybrid KO AGS phenotype more accurately shows the severity of the *fmr1-tm1Cgr* AGS allele, as elevations of AGS sensitivity in inbred FVB KO and C57 KO backgrounds are likely to be due to other recessive AGS loci in those strains. Furthermore, these data indicate that the *fmr1-tm1Cgr* allele appears to be a very significant determinant of AGS in its own right – more potent than the FVB AGS allele under our conditions.

One difference between our data and that reported previously (Chen & Toth 2001) is that in the earlier study no AGS was seen before 10 weeks of age and never in FVB WT animals of any age, while we saw very high levels of seizures in FVB KO animals by 3 weeks of age, as did Musumeci *et al* (2000). We, like Musumeci *et al*., used a 120 dB stimulus while Chen & Toth used one of 115 dB and possibly a broader frequency range which was described as white noise. Thus the difference in results may be a function of protocol or equipment; alternatively, the early auditory environment is also known to have an effect on subsequent AGS sensitivity in rodents. A further difference noted is that no reduced susceptibility of a 129 $\times$ FVB cross was observed to audiogenic seizures, leading to the conclusion that genetic modifiers do not play a major role in the *fmr1* AGS phenotype (Chen & Toth 2001). However, our F1 hybrid data argue that such modifiers must play a substantial role. The hybrid KO data presented here indicate that the *fmr1-tm1Cgr* allele has a greater effect on AGS susceptibility than the background modifier present in the WT FVB or C57 strains.

Differential

There are several reasons why *fmr1-tm1Cgr* mice might show a phenotype distinct from that seen in humans with FXS. It is unlikely that these mice, or even humans with FXS, suffer a significant global biochemical deficit, such as a near complete absence of synaptic polyribosomes and therefore protein translation at the base of dendritic spines in response to neurotransmitter stimulation (Greenough *et al*. 2001), since basic neurologic functions are not obviously affected.

Species specific compensation

It is possible, however, that genetic compensation can differ between species, such that the phenotype of a mutation in one would not be of comparable severity to that in another. For example, mutation of the *HEXA* gene in humans causes the lethal Tay-Sachs disease, while mutation in the related *HEXB* gene leads to the milder Sandhoff disease. It appears that *HEXB* may be compensating for *HEXA* more in mice than in humans such that *HEXA* KO mice have an unexpectedly mild, and *HEXB* mice an unexpectedly severe phenotype, relative to what is seen for mutations in these genes in humans (Crawley 1996). The same could be true for the two highly conserved paralogs of FMRP (FXR1P and FXR2P), which might compensate more for FMRP loss in mice than in humans. Furthermore, mutation of a gene such as *Fmr1* that affects the expression of other genes might lead to amplification of any differences in compensation between species, and even between inbred strains.

Developmental timing

Given that what are interpreted as cognitive effects in behavioral tests even in the inbred KO strains are so mild, it seems reasonable to postulate that the mouse species in general is simply less susceptible to the detrimental effects of reduced FMRP than the human species. Such a difference could be due to differences in developmental timing. Interestingly, in one careful examination of the effects of *fmr1-tm1Cgr* dendritic spines, it was found that the effects of the mutation were only seen for the first month of life (Nimchinsky *et al*. 2001).

Anatomic, evolutionary or functional differences

Another possible explanation of the perceived differences in the effect of mutation of *Fmr1* in the mouse and human is that a key structure affected in humans is not present or used in a comparable way in the mouse. For example, humans may use internal verbal recall for some forms of working memory, while such a process in non-human animals is to date unknown. A mild deficit in higher cortical functions could produce what in humans is considered mental retardation, while in mice such functions might not be relevant. Indeed, within the view that the defect in FXS might actually be relatively 'mild', it should be considered that the mouse neocortex is much smaller than the human relative to the rest of the body, with a difference in encephalization quotient of more than an order of magnitude (Rogers 1998). If the defect in FXS neural function is actually quite small for a single neuron *in vivo*, then the overall effect of this defect may be related to the relative size and importance of the tissue involved. If the neurons in the neocortex are affected by FMRP loss, then the more complex neocortex (human) may be more affected in its operation than the less complex (mouse). Consistent with an important role for neocortical function in human FXS is its impact on language and executive function (Fisch *et al*. 1999a; Munir *et al*. 2000b). Furthermore, a cortical specific deficit in *Glu*RI and LTP of *fmr1-tm1Cgr* mice was recently reported (Li *et al*. 2002). No defect was found in the hippocampus or cerebellum. If the above scenario is valid, then the magnified effect of FMRP loss in humans relative to mice would be indicative of a slight biochemical defect which has been amplified in the enlarged human neocortex. If so, it might be the case that correction or bypass of such a defect would allow the affected tissue to respond in a more effective manner.

Test stringency

It seems unlikely that we are seeing inadequate test stringency for all tests, particularly since several of the tests employed are similar to those for which large performance deficits are seen in people with FXS. In several of the mazes used here, other known mouse models of mental retardation, such as for phenylketonuria (PKU), do show performance at least two standard errors below that of the WT mean (Hodges 1996), as would be expected from the definition of human mental retardation. The olfactory task we employed was stringent enough to detect clear difference between two strain backgrounds (FVB and F1 hybrid). Indeed, the 2-sequence olfactory working memory task employed in this study is so stringent that performance was significantly improved by holding the second odor constant (Katz *et al*. 2003). Nonetheless, even the FVB WT and KO mice were able to improve their perfect choice responses more than threefold within 65 trials. Furthermore, constantly changing odor values were designed to allow learning set formation to be assessed. Learning set formation can imply rule or strategy-based higher cognitive function, as opposed to memorization of associations. Although we do not rule out the presence of procedural responses (Katz *et al*. 2003), the olfactory task includes reversals of odor values and delays, both of which require neocortical inputs. Nonetheless, additional tests of varying difficulty, as well as of motivation (Strupp & Levitsky 1990), will be needed to determine whether the *fmr1-tmCgr* mouse model has a cognitive deficit more consistent with human MR than has been noted to date.

Strain and modifier effects

Different degrees of allelic penetrance, sex chromosome origin, and epigenetic effects could account for differences observed among strains in various studies. For example, the origin of the X and Y chromosomes in an F1 hybrid can be important since much of the testing is performed in male mice. Parental origin reversals in hybrids can be used to assess whether epigenetic or sex chromosomal effects are contributing to a phenotype. Another consideration for studies employing F1 hybrid mice is the degree to which inbred parental strains are related and thereby would be likely to share alleles. For example, in one analysis of 565 allele fixations, C57BL differed by over 120 differences from outbred Swiss mice, from which the FVB/NJs were derived. In contrast, C57BL and 129 strains differed by approximately 75 fixations (Atchley & Fitch 1991). Inbreeding coefficients for various F1 hybrid strains could be calculated using such data in order to assess how effective the cross should be in reducing the presence of homozygous recessive loci. It should also be noted that many alleles show varying degrees of dominance (complete or incomplete) that may also contribute to differences in phenotype, even between F1 hybrid strains. Nevertheless, the AGS, startle and olfactory learning comparisons presented here argue that reducing the effects of recessive alleles will help reduce strain specific confounds such as seizure sensitivity, deafness and blindness. An additional consideration is that the X chromosomal region of the C57 backcrossed heterozygous *fmr1-tm1Cgr* females used in many published experiments possibly harbors neighboring alleles from the original strain 129 genome in which *Fmr1* gene targeting occurred, but the same region in the littermate controls would be of C57 origin. It has been

estimated that after 12 backcrosses, 1% of the original 129 genome might remain associated with the targeted mutation (Gerlai 1996), which in turn could represent as many as 100 genes expressed in the CNS (Lathe 1996). Rescue experiments with transgenic mice containing a human *FMR1* PAC are currently underway to further assess the actual effect of the *fmr1-tm1Cgr* allele on positive test results (M. Rammal, Q.J. Yan and R.P. Bauchwitz, unpublished data).

The data presented here also raise the question of why the hybrid strains were less affected in the most robust phenotype, seizure susceptibility, and in particular status epilepticus. The seemingly reduced phenotypic effect of this allele in the hybrid background suggests that genetic modifier loci may be involved, as previously suggested (Dobkin *et al.* 2000). Some such loci, however, may be of minimal relevance to FXS, such as retinal degeneration in FVB/NJ mice (Pittler & Baehr 1991), elevated seizure sensitivity of the same strain (Goelz *et al.* 1998) and hearing deficits of the C57BL/6J strain (Johnson *et al.* 1997). Thus, by combining the strains, we are able to use animals with presumably more normal sight, hearing and seizure thresholds, which was our intent in using the hybrids as a model. However, it may also be that C57 × FVB hybrid mice have a genetic makeup which makes them more resistant to the effects of the *fmr1-tm1Cgr* mutation for more fundamental reasons. This possibility would be supported if a molecular null mouse model (i.e. with no possibility of *Fmr1* leakiness during development) were to have an enhanced effect in the inbred strains but still no effect in the hybrids.

Molecular leakiness

Finally, we have presented evidence that the *fmr1-tm1Cgr* allele is not a molecular null. Our data indicate that the *fmr1-tm1Cgr* insertion creates a splicing mutation which leads to aberrantly spliced transcripts (Nissim-Rafinia & Kerem 2002). The existence of *Fmr1* mRNA in the *fmr1-tm1Cgr* mouse is tenable since the promoter has been left intact. With specific reference to the *fmr1-tm1Cgr* mRNA transcript with an open reading frame extending the length of the gene, we note that the addition of the predicted 51 amino acids would make the resulting FMRP's chimeric proteins. There is, however, no reason to expect such proteins to be inactive. There is no proven function for the disrupted exon 5. Furthermore, the most prevalent isoforms of FMRP are over 600 amino acids in length, so an additional 50 amino acids (~5.5 kDa) would represent an approximately 8% increase in size. Green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, is a 238 amino acid protein with a molecular weight of 27 kDa. It is routinely used to make tagged, functional fusion proteins *in vivo*, including with internal insertions in the recipient peptide. Therefore, there is no *a priori* reason why the presence of a small increase in the number of amino acids in this region of FMRP would necessarily inactivate it. We conclude that firstly, *Fmr1* mRNA is being transcribed from the *fmr1-tm1Cgr* allele and secondly, at least one of the novel splice products from *fmr1-tm1Cgr* contains an open reading frame that could produce an active form of FMRP. We are unaware of any precedent for such an mRNA not to be translated.

A potentially instructive example of the large effects that having residual gene expression can have comes from examination of two KO mouse models of the *MAP1B* gene. *MAP1B* codes for one of the high molecular weight microtubule associated proteins present in neurons. *MAP1B* has been implicated as affected by deficient FMRP levels (Brown *et al.* 2001; Zalfa *et al.* 2003). The first disruption of *MAP1B* in the mouse, *Map1b571* (Edelmann *et al.* 1996) was made in a manner very similar to that of the *fmr1-tm1Cgr* mouse in that a PGKneo cassette was inserted into an exon in an orientation opposite to transcription. As we have shown here for *fmr1-tm1Cgr*, mRNA is expressed from the *Map1b571* allele, and truncated products are predicted (but not observed by Western blotting). The phenotype of *Map1b571* mice was quite severe, leading to death of homozygotes *in utero*. *Map1b571* heterozygous showed ataxia and Purkinje cell deficits. In contrast, when a more likely true molecular null mouse model of *MAP1B* was subsequently described, *Map1bR21* (Takei *et al.* 1997), heterozygotes were normal, while homozygotes had reduced brain size and development, but no Purkinje cell deficits. As the *Map1b571* phenotype differed significantly from that of *Map1bR21*, it was concluded that something expressed from the *Map1b517* locus was having a biologic effect, perhaps acting as a dominant negative. It other similar cases, it has been proposed that the PGK promoter sequences left within a disrupted allele may have had long range effects on expression of neighboring genes (Bultman & Magnuson 2000).

Thus, it may be that low levels of FMRP or fragments thereof, or even the *Fmr1* mRNA itself, can prevent the development of serious cognitive deficit in the mouse. If so, then similar low levels of *FMR1* products might have a beneficial effect in the human FXS. We emphasize, however, that the *fmr1-tm1Cgr* mouse is not equivalent to a WT and does show some behavioral differences as well as physical differences, most notably enlarged testes, enhanced susceptibility to acoustically generated seizures and altered open field behavior. We propose that the *fmr1-tm1Cgr* phenotype is possibly hypomorphic with respect to the syndrome seen in humans. Quantitative changes in neuronal protein levels are known to be capable of substantial impact – for example, reducing NMDAR levels in mice to less than 5% of normal leads to schizophrenic-like symptoms, while true nulls die perinatally (Mohn *et al.* 1999).

Conclusion

The data presented here suggest that *fmr1-tm1Cgr* mice do not have grossly obvious deficits in working memory (novel object, radial maze and olfactory sequence tests). A difference

in Barnes maze probe trial is reported, although a general deficit in spatial long-term memory is not seen, as assessed in the MWM. *fmr1-tm1Cgr* mice do cross into and spend more time in the center of an open field. However, EPM data do not support a simple interpretation that the mice are less anxious than WT littermates. Differences in search tendencies and/or exploratory behavior may underlie the Barnes maze and open field findings. Finally, the *fmr1-tm1Cgr* mouse is not a molecular null. It produces substantial *Fmr1* mRNA, and may even express low levels of FMRP. This could contribute to the unusually mild cognitive phenotype. Nonetheless, the data show that *fmr1-tm1Cgr* mice are not equivalent to WT and therefore may have continuing value as an assay for the disorder.

The ultimate reason for the difference in phenotype of human and mouse mutations in *FMR1* remains uncertain. While we favor the possibility that a relatively mild biochemical deficit leads to noticeable cortical dysfunction in FXS humans but much less so in mice, this will not be known until the effects of potential leakiness of the *fmr1-tm1Cgr* allele are determined. It will be important to compare the phenotype of the *fmr1-tm1Cgr* mouse to a true molecular null, and if possible, an expanded repeat mouse model of *Fmr1*, in order to more definitively assess the meaning of the phenotypes observed for *fmr1-tm1Cgr* mice.

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