Published in final edited form as:

Autism Res. 2011 February ; 4(1): 40–56. doi:10.1002/aur.168.

Modifying Behavioral Phenotypes in *Fmr1* KO Mice: Genetic Background Differences Reveal Autistic-Like Responses

Corinne M. Spencer¹, Olga Alekseyenko¹, Shannon M. Hamilton², Alexia M. Thomas², Ekaterina Serysheva¹, Lisa A. Yuva-Paylor¹, and Richard Paylor^{1,2,*}

¹ Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030 USA

² Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030 USA

Scientific Abstract

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability in humans. In addition to cognitive impairment, patients may exhibit hyperactivity, attention deficits, social difficulties and anxiety, and autistic-like behaviors. The degree to which patients display these behaviors varies considerably and is influenced by family history, suggesting that genetic modifiers play a role in the expression of behaviors in FXS. Several studies have examined behavior in a mouse model of FXS in which the *Fmr1* gene has been ablated. Most of those studies were done in *Fmr1* knockout mice on a pure C57BL/6 or FVB strain background. To gain a better understanding of the effects of genetic background on behaviors resulting from the loss of *Fmr1* gene expression, we generated F1 hybrid lines from female *Fmr1* heterozygous mice on a pure C57BL/6J background bred with male *Fmr1* wild-type mice of various background strains (A/J, DBA/2J, FVB/NJ, 129S1/SvImJ and CD-1). Male *Fmr1* knockout and wild-type littermates from each line were examined in an extensive behavioral test battery. Results clearly indicate that multiple behavioral responses are dependent on genetic background, including autistic-like traits that are present on limited genetic backgrounds. This approach has allowed us to identify improved models for different behavioral symptoms present in FXS including autistic-like traits.

Keywords

fragile X syndrome; autism; genetic; behavior; animal model; mouse model

Introduction

Fragile X syndrome (FXS) is widely acknowledged as the most common form of inherited intellectual disability (ID). The prevalence of FXS is estimated to be 1/4000 males and 1/8000 females, which accounts for approximately one-third of all X-linked ID cases (Sherman, 2002). The FXS phenotype is often described as clinically indistinct due to the broad spectrum of involvement of the various physical, cognitive, and behavioral abnormalities associated with the syndrome. What is often less appreciated, but which may be more critical to the overall quality of life, is the fact that individuals with FXS have several other behavioral abnormalities including: attention deficit, hyperactivity/hyperkinesis, anxiety, depression, irritability, mania, obsessive-compulsive behavior, aggression, and self-injurious behavior (Hagerman, 2002). FXS patients are also hypersensitive to many different sensory stimuli. Interestingly, a number of autistic-like

*Correspondence: Richard Paylor, rpaylor@bcm.edu, Tel: 713-798-6124, Fax: 713-798-7773.

behaviors have been associated with FXS, including poor eye contact, tactile defensiveness, hand biting, hand flapping, and perseveration of speech and behavior (Hagerman, 2002). An estimated 21–50% of individuals with FXS meet diagnostic criteria for autistic disorder, displaying social difficulty, communication problems and perseverative or repetitive behaviors (Moss & Howlin, 2009). Like many complex genetic syndromes, there is significant variability in the expression of these behavioral abnormalities (Hessl *et al.*, 2001; Hagerman, 2002), i.e. not all individuals display the same abnormal responses, nor do they express abnormal behaviors to the same degree of severity. The nature of the variability, including the presence of autism in a percentage of the individuals with FXS, is most likely the result of differences in genetic background (G), differences in prenatal and postnatal environment (E), and G X E interactions. In addition, epigenetic factors likely contribute to variations in the expression of behavioral abnormalities observed in FXS. Unfortunately, it is very difficult to examine the role of each of these underlying factors contributing to variability in FXS. Therefore, we have turned to the use of the *Fmr1*KO mouse model of FXS to systematically evaluate the role of genetic background in behavioral variability associated with *Fmr1* loss of function. The genomic structure of the mouse and human *FMRI* genes has been characterized and indicates 95% sequence identity, 97% amino acid identity, and striking structural conservation (Ashley *et al.*, 1993). The high degree of homology and the similarity of expression between the human and mouse genes (Abitbol *et al.*, 1993; Hinds *et al.*, 1993) make the mouse a good model system in which to study the role of genetic background on the variation among behavioral responses of individuals with FXS.

There is no doubt that genetic background significantly alters baseline and drug-treated behavioral responses in mice. Multiple inbred strain surveys clearly demonstrate that different strains of mice respond differently in a variety of behavioral settings (e.g. Crawley *et al.*, 1997; Bothe *et al.*, 2004). For example, Logue and colleagues (1997) have shown that there are tremendous differences in performance on learning and memory tasks among various inbred and F1 hybrid strains. Similarly, we have recently shown that behavioral responses differ among inbred strains on both behavioral and physiological indicators of anxiety (Bouwknicht & Paylor, 2002). In addition, numerous investigators have demonstrated that genetic background has a significant impact on responses to various drugs such as ethanol and nicotine (e.g. Crabbe *et al.*, 1982; Marks *et al.*, 1989). A review of various behavioral responses that differ among inbred strains of mice can be found in Crawley *et al.* (1997). Taken together the studies with inbred and F1 hybrid strains clearly demonstrate that differences in genetic background can have a dramatic impact on behavioral responses of mice.

Investigators interested in either systematically investigating the role of genetic background or controlling for genetic background routinely choose one or two inbred strains such as C57BL/6 and backcross the targeted mutation 10 generations to reach congenicity (i.e. stable genetic background). Often, researchers create an F1 hybrid between two congenic lines (see Banbury Conference on Genetic Background in Mice, 1997). There are a few reports of mutations on 2–3 genetic backgrounds showing that behavioral phenotype is affected by genetic background (e.g. Holmes *et al.*, 2002; Moy *et al.*, 2009). However, these studies were not able to control for two variables known to exert significant effects on behavior- namely maternal behavior and differences in the flanking regions surrounding the mutation. Maternal behavior, which is influenced by the genetic background of the mother, influences the environment in which the offspring are raised (eg. Weller *et al.*, 2003; Zupan & Toth, 2008). The flanking region will co-segregate with the mutation; however, in the course of backcrossing to a new background strain the flanking region will also be altered, thus increasing variability in that region (Crusio, 2004; Schalkwyk *et al.*, 2007).

Recent studies with *Fmr1*KO mice have shown that behavioral phenotypes of *Fmr1*KO mice can be dependent on genetic background. Paradee and colleagues (1999) have shown that *Fmr1*KO mice on a pure C57BL/6 background have normal conditioned fear, however, *Fmr1*KO mice on a C57 X FVB F1 background appear to have impaired conditioned fear. Prepulse inhibition (PPI) is enhanced in *Fmr1*KO mice on a pure C57 background, but normal on an F1 background (Nielsen *et al.*, 2002). Recently, Moy and colleagues (2009) showed that low sociability in *Fmr1*KO mice is observed in *Fmr1*KO mice on a FVB genetic background and not a C57BL/6 background.

Although the behavioral findings reported above support the notion that the behavioral responses of mutant mice, including *Fmr1*KO mice, are dependent on genetic background they are limited by several important factors. First, each study evaluated a limited number of different genetic backgrounds, which does not necessarily give an accurate assessment of the impact of genetic background on the expression of behavioral responses in mutant mice. Second, the breeding schemes used for many of these studies do not control for differences in maternal factors. It is possible that differences in the behavior of mutant mice on different genetic backgrounds are partially due to genetic differences in the mother, which can affect maternal factors that alter the behavioral responses of her offspring (e.g. Francis *et al.*, 2003). Third, most studies comparing different genetic backgrounds have not necessarily controlled for possible differential chromosomal segments flanking any mutation that could be varied on different genetic backgrounds. Finally, the extent of the behavioral assessments for most of these studies was limited, so it is difficult to know the extent of the genetic background effects on a wide range of behavioral responses. For the studies described here, we utilized a breeding scheme that allowed us to evaluate multiple genetic backgrounds, while controlling for maternal factors and differential chromosomal segment flanking regions, and employed an extensive behavioral test battery to make a comprehensive assessment of multiple behavioral responses of the various lines of *Fmr1*KO mice. The test battery used in previous studies on Fragile X mouse models from our laboratory (Peier *et al.*, 2000; Spencer *et al.*, 2006; Zang *et al.*, 2009) was modified to include additional assays of mouse behaviors relevant to autism, such as repetitive behavior (Thomas *et al.*, 2009) and social behavior (Spencer *et al.*, 2005; Spencer *et al.*, 2008).

The current study clearly demonstrates that: (1) genetic background not only influences behavioral responses in mutant mice, but one could argue that it is a determining factor, (2) the presence of ‘autism-related’ phenotypes is observed in limited genetic backgrounds, and (3) genetic background can be used as an important tool to systematically model and study the role of ‘other genes’ in contributing to the variation in responses among individuals with neurodevelopmental disorders.

Methods and procedures

Animals

Male *Fmr1*KO and wild-type (WT) littermates from a congenic (N11-N13) C57BL/6J background and five F1 hybrid lines were generated by crossing female *Fmr1* heterozygous mice on a congenic C57BL/6J background with male wild-type mice of the following background strains: A/J, DBA/2J, FVB/NJ, 129S1/SvImJ and CD-1. For ease of identification, the genetic backgrounds will be referred to as B6 (for the congenic line), B6A, B6D2, B6F, B6S1 and B6CD. Female parental stock was bred at Baylor College of Medicine and genotyped as described previously (Spencer *et al.*, 2005). Male parental stock was obtained from the Jackson Laboratory (Bar Harbor, ME). The mice for this study were bred at Baylor College of Medicine and housed 2–5 per cage in a room with a 12 hr light: 12 hr dark schedule (lights on at 06:00am). All mice had access to food and water *ad libitum*. All animal care and behavioral testing procedures were approved by the Baylor College of

Medicine Animal Care and Use Committee and followed the NIH Guidelines, “Guide for the care and use of laboratory animals”.

Testing procedures

Mice were approximately 2–3 months of age when behavioral phenotyping was initiated. In general, behavioral testing was performed between 8 AM and 3 PM by experimenters who were blinded to the genotypes at the time of testing. Male mice (B6: 15 KO, 15 WT; B6A: 15 KO, 15 WT; B6D2: 17 KO, 17 WT; B6F: 15 KO, 16 WT; B6S1: 15 KO, 15 WT; B6CD: 15 KO, 16 WT) were assessed on the following tests in order: (1) open-field test, (2) light-dark exploration test, (3) marble-burying test, (4) accelerating rotarod test, (5) tail suspension, (6) acoustic startle response and prepulse inhibition (PPI) of the acoustic startle response, (7) acoustic startle habituation, (8) conditioned fear, (9) hotplate, (10) novel food response, (11) partition test, and (12) social interaction test. Tests were performed as described below, with a 1–3 day inter-test interval (Paylor *et al.*, 2006) except for a 7-day interval between the hotplate and novel food tests. The complete test battery took 28 days from start to finish.

Open Field Exploration

Open field activity was measured in the VersaMax Animal Activity Monitoring System (AccuScan Instruments, Columbus, OH). Mice were placed into the center of a clear acrylic (40 cm × 40 cm × 30 cm) chamber under approximately 800 lux of illumination and 55 dB of white noise. Data were collected in two-min intervals for each mouse for 30 min on each of two consecutive days. The center distance: total distance ratio, which is used as a measure of anxiety-like behavior (Crawley, 1989), was calculated by dividing the center distance by the total distance. Open-field activity data were pooled into six 10-min intervals and analyzed using a three-way (genotype × background × interval) analysis of variance (ANOVA) with repeated measures.

Light–Dark Box Exploration

One day after the open-field test, anxiety-related responses were assessed in the light-dark box. The apparatus is composed of two adjoining chambers: a dark enclosed chamber composed of black plexiglas (15 cm × 21 cm × 21 cm) and a larger chamber (30 cm × 21 cm × 21 cm) with three clear plexiglas walls and an open top. The two chambers are connected by a small opening. The larger chamber was brightly illuminated (800 lux), and white noise was present at approximately 55 dB. Mice were placed into the illuminated side and allowed to explore freely for 10 min. A hand-held event recorder (Psion Workabout mx, Psion Teklogix) together with the OBSERVER program (Noldus Information Technologies) was used to score the number and latency of entries, and time spent in each compartment. An entry was defined as the mouse placing all 4 feet into the zone.

Marble bury test

The marble bury test was used to evaluate repetitive digging behavior (Thomas *et al.*, 2009). One day after the light-dark test, mice were placed into a standard mouse cage containing clean corncob bedding (approximately 5 cm deep) and 20 marbles arranged in a 4 × 5 array on top of the bedding. Mice were placed into the cage onto approximately 2 in of space that was left clear of marbles at one end of the cage, the cage was then covered with a filter-top lid and the mice were left undisturbed for 30 min. At the end of the test, mice were carefully removed and the number of marbles that were more than 50% buried was recorded.

Rotarod test

Using an accelerating rotarod (UGO Basile, Varese, Italy), motor coordination and skill learning were tested 3 days after the marble bury test. Mice were placed on the rotating drum, which accelerated from 4 to 40 rpm over a 5 min period. Time spent walking on top of the rod before either falling off the rod or slipping and riding completely around the rod was recorded. Mice were given four trials on two consecutive days with a 30–60 min inter-trial rest interval. Each trial had a maximum time of 300 seconds (5 min).

Tail Suspension Test

Immobility time was determined by an automated TST system (Med Associates, St. Albans, VT) one day following the rotarod test. The mouse was taped by the tail to an aluminum bar connected to a strain gauge and suspended for 6 min. Immobility time was defined as the duration in which the force of the mouse's movements fell below the threshold level (<5) for at least 100 msec.

Acoustic startle and prepulse inhibition of acoustic startle

One day later, acoustic startle responses were measured using the SR-Lab System (San Diego Instruments, San Diego, CA) as previously described (Paylor *et al.*, 2008). Mice were placed in a Plexiglas cylinder within a sound-attenuated chamber and acclimated to background white noise (approx. 70 dB) for 5 min prior to beginning the test session. There were seven trial types within the test session. One trial type consisting of no stimulus was presented to measure baseline movement in the cylinder. Maximum startle response was measured in a trial type consisting of the startle stimulus alone (40 ms, 120 dB). In the remaining trial types, five different 20 ms prepulse sounds (74, 78, 82, 86, or 90 dB) were presented 100 ms before the startle stimulus. Each trial type was presented six times, once per block of seven trials in pseudorandom order, with an inter-trial interval of 10 to 20 sec. The startle response was recorded every 1 ms during a 65 ms period that followed the onset of the startle stimulus. The maximum startle amplitude during this period was used as the dependent variable. For each subject, the startle amplitude for each trial type was averaged across the 6 trials. Percent prepulse inhibition of the startle response was calculated as $100 - [(startle\ response\ on\ acoustic\ prepulse\ plus\ startle\ stimulus\ trials / startle\ response\ alone\ trials) \times 100]$ for each subject. Acoustic response amplitude data were analyzed using a two-way (genotype $\times$ background) ANOVA. For prepulse inhibition, the average of the three lowest prepulse levels (74 dB, 78 dB, 82 dB) was taken. The data for mice that did not meet our criterion for minimum startle response to the 120 dB sound stimulus (100) were not included in the analysis.

Startle Habituation

One day after the PPI test, habituation of acoustic startle responses was measured using the SR-Lab System as described above. Following a 5 min acclimation period with background 70 dB white noise, the startle stimulus (40 ms, 120 dB) was presented 100 times. Data were combined into 10 bins.

Pavlovian conditioned fear

Three days after startle habituation, mice were trained and tested in a conditioned fear paradigm. On the training day, mice were placed into the test chamber (Med Associates, St. Albans, VT) and allowed to explore for 2 min. The conditioned stimulus (CS, a white noise 80 dB sound) was then presented for 30 sec and was followed immediately by a mild foot shock (2 sec, 0.7 mA) that served as the unconditioned stimulus (US). After an additional 2 min the mice received a second CS-US pairing and were then returned to their homecages. Timing of CS and US presentations were controlled by the FreezeFrame monitor system

(San Diego Instruments, San Diego, CA) while freezing behavior was scored every 10 sec by an experimenter who was blinded to the genotypes. Percent freezing was determined by the percentage of 10-sec intervals in which freezing was observed. Mice that did not show a response to the foot shock (typically run, jump or vocalize) during the conditioning procedure were not included in the final analysis.

Approximately 24h after training mice were tested for contextual fear conditioning by placing them back into the original test chamber for 5 min and assessing freezing behavior every 10 sec. One to two hours later, mice were tested for responses to the auditory CS in a new chamber with different contextual cues. Black Plexiglas inserts were placed on the floor and sides of the chamber to alter the shape, texture and color of the chamber and vanilla extract was placed in the chamber behind the insert to alter the odor. Mice were brought into the testing room under dim red light conditions in transfer cages that contained paper towels instead of bedding and then placed into this new chamber. Freezing was recorded as described for 3 min during a “pre-CS” phase and then for another 3 min while the auditory CS was presented. Data for the CS test were calculated as the percent freezing during the CS minus percent freezing in the pre-CS phase.

Hotplate test

Analgesia-related responses were assessed using a hotplate apparatus (Columbus Instruments, Columbus, OH). Subjects were placed one at a time onto the hotplate preheated to $55^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and the time to first show a hindlimb response was recorded. Mice were removed immediately after showing a response (typically licking or shaking the hindpaw, or jumping).

Novel food response

On the third day of single-housing before the partition test, subjects were given access to approx. 3 g of Kellogg’s Nutri-Grain blueberry bar for 20 min. Remaining food was weighed to determine amount consumed.

Social Partition test

A partition test was used to evaluate social behaviors in mice during a non-contact version of a social interaction test in a familiar test environment. The method used was adapted from Kudryavtseva (1994) to include an assessment for basic social recognition in addition to assessment of social interest (Spencer *et al.*, 2005). Subjects were housed individually for 4 days (96 hours) in standard cages. At 2:00 PM on the day before the test, each subject was placed in one side of a standard cage divided in half by a clear perforated (0.6 cm diameter holes) partition. A partner mouse (C57BL/6J; gender-, age- and weight-matched) was placed in the side opposite the experimental mouse. On the day of the test (10:00am–2:00 pm), mice were given the partition test in three 5 min phases. First, approaches and time spent at the partition by the experimental mouse were measured with the original overnight partner. Second, the approaches and time spent at the partition by the experimental mouse were measured with a new partner. Third, the behavior of the experimental animal was scored with the original partner. To normalize for each experimental animal, time spent at the partition with the unfamiliar partner was calculated as a percentage of the time spent at the partition with the familiar overnight partner. Paired t-tests were performed for *post hoc* comparisons in order to assess social recognition for each genotype in each background strain.

Social interaction test

After the partition test, subjects continued to be housed in the partitioned cages across from their original partners. Three days after the partition test, a direct social interaction test was conducted. The standard filtered cage lid was replaced with a clear perforated lid and mice were acclimated for 5 min. Then the partition was removed and the mice were videotaped from above for 10 min. Behavioral responses were later scored as described (Spencer *et al.*, 2005) from the videotapes by an observer blinded to the genotypes of the mice using a Psion hand-held computer with the Noldus OBSERVER program. Scored behaviors included the following, grouped into three major categories: Active social behavior is behavior initiated by the subject toward its partner: 1) Anogenital sniffing; 2) Non-anogenital sniffing; 3) Direct aggressive attacks, such as biting or kicking; 4) Lateral threats; 5) Tail rattling; 6) Chasing; 7) Aggressive grooming; 8) Wrestling/boxing -fighting between mice when they are equal and it is impossible to tell the direction of the behavior. Passive social behavior is behavior that occurs as a reaction to the active/aggressive behavior of the partner mouse toward the subject mouse: 9) Receptive -when the subject accepts the sniffing from the partner but doesn't show signs of defensive or submissive behavior; 10) Escape, such as leaping or fleeing; 11) Freezing or immobilization; 12) Defeat postures (passive defense); 13) Upright defense (active defense) - when subject rears up on its hindlegs, rotating the upper torso toward the aggressive opponent, sometimes with kicking movements of forelimbs. Scored non-social behaviors included: 14) Exploring the cage; 15) Rearing in any part of the cage; 16) Rest - sitting, sleeping or eating; 17) Digging up and scattering the bedding; 18) Self-groom -body care activities. Data were analyzed within the broad behavior categories.

Isolation-induced ultrasonic vocalization (USV) in pups

A total of 79 pups from 9 B6D2 litters (litter size = 6–12) were obtained and USVs were assessed on postnatal days (PND) 3–14. Each litter was first moved with its nesting material from its home cage to a holding container. Then each pup was removed one at a time and placed into a sound-attenuating chamber for 2 min while its USVs (57–77 kHz) were recorded (BAT Detector mini-3, Noldus UltraVox 2.0). The full ultrasonic range was monitored simultaneously using a modified frequency division detector (Pettersson Ultrasound Detector D230). Pups were identified by plantar surface tattoos, which were placed after testing on PND3, and genotyped after weaning. Data from 25 male *Fmr1*KO and 13 male WT mice were analyzed by two-way (genotype $\times$ day) ANOVA with repeated measures.

Statistical analyses

Unless otherwise noted, data were analyzed by two-way ANOVA (background $\times$ genotype) or three-way (background $\times$ genotype $\times$ repeated measure) ANOVA using the SPSS statistical software package (SPSS, Chicago, IL). Regardless of whether there was a significant strain $\times$ genotype interaction, one-way ANOVA was performed to compare KO and WT mice within each line. The level of significance was set at $p \leq 0.05$.

Results

For all assays, results are reported first for overall effect of the *Fmr1* null mutation across all six genetic backgrounds, and then for differences between *Fmr1*KO and WT littermates within each background. As expected, a main effect of genetic background was observed in all measures reported (see Table 1 for p values) with the exception of data that was normalized.

Locomotor activity/exploration

Most males with FXS exhibit hyperactivity and/or hyperarousal (Hagerman, 2002). *Fmr1*KO mice were tested in the open field for 30 min on two consecutive days (6 × 10 min intervals) to assess exploratory activity and habituation to a novel environment. There was a main effect of genotype for total distance traveled ($F(1, 175) = 16.467, p < 0.0005$), with *Fmr1*KO mice traveling a greater distance than their WT littermates (Figure 1A). Significant differences (KO>WT) were observed in three of the genetic backgrounds: B6 ($p=0.006$), B6D2 ($p=0.001$) and B6F ($p=0.001$). No significant differences were observed in the B6A, B6S1 or B6CD backgrounds.

Looking at distance traveled during the 10-min intervals as a measure of habituation to a novel environment, there was no overall interval × genotype interaction ($F(5, 875) = 0.904, p=0.478$). However, in the B6F background there was a significant interval × genotype interaction on the first day (data not shown; $F(2, 60) = 4.672, p=0.013$). B6F-*Fmr1*WT mice showed significant decreases in distance traveled from the first to the second interval ($p < 0.005$) and from the second to the third interval ($p=0.002$). However, B6F-*Fmr1*KO mice did not show these decreases over time ($p=0.121$ and $p=0.063$, respectively), suggesting reduced habituation to the novel environment. On Day 2, there was no difference between *Fmr1*KO and WT on this background.

There was a main effect of genotype in vertical activity ($F(1, 175) = 4.448, p=0.036$), with *Fmr1*KO mice showing less vertical activity (rearing) than their WT littermates (Figure 1B). Specifically, *Fmr1*KO mice had less vertical activity in the B6CD and B6S1 backgrounds ($p=0.042$ and $p=0.045$). In the B6D2 background, however, *Fmr1*KO mice showed greater vertical activity than WT littermates ($p=0.007$).

Motor coordination and skill learning

Mice were tested on an accelerating rotarod for eight trials over two consecutive days for motor coordination and skill learning. There was no main effect of genotype ($F(1, 174) = 0.316, p=0.575$) for time spent walking on top of the rotarod. *Fmr1*KO mice on the B6S1 background spent more time on the rotarod than their WT littermates ($p=0.018$). There was a trend to a difference between WT and *Fmr1*KO mice on the B6D2 background ($p=0.079$), and no difference between genotypes on the B6, B6A, B6F or B6CD backgrounds (data not shown).

Stereotyped behavior

Studies have reported that 85–100% of males with the full mutation of FXS demonstrate perseverative or stereotypic behaviors, especially hand-flapping and hand-biting (Hagerman, 2002). In the open field, stereotypy is measured by repeated patterns of beambreaks. A main effect of genotype was observed for stereotypy in the open field ($F(1, 175) = 6.917, p=0.009$) with *Fmr1*KO mice showing increased stereotypic activity (Figure 2A). When examining individual backgrounds, only the *Fmr1*KO mice on the B6D2 background exhibited greater stereotypy than WT littermates ($p < 0.0005$).

The marble bury test has been suggested as a rodent behavioral model of obsessive-compulsive disorder (OCD) (Njung'e & Handley, 1991). Recently, our laboratory has shown that marble bury is a good measure of repetitive, perseverative digging (Thomas *et al.*, 2009). The number of marbles buried during 30 min in a clean cage with excess bedding material was determined. There was no main effect of genotype ($F(1, 175) = 1.385, p=0.241$) in marble-burying; however, there was a significant effect in one of the lines (Figure 2B). *Fmr1*KO mice in the B6D2 background buried more marbles than their WT littermates ($p=0.042$).

Anxiety- and depression-related behavior

Mice were tested in three assays to examine anxiety-related behavior. As expected significant main effects of background strain were evident in all anxiety-related tests (see Table 1). In the open field, the center distance: total distance ratio is used as an index of anxiety-like behavior (Crawley, 1989). There was an overall effect of genotype ($F(1, 175) = 7.973, p = 0.005$) in which *Fmr1*KO mice traveled more in the center of the arena relative to WT littermates (Figure 3A). When examining individual backgrounds, only *Fmr1*KO on the B6A and B6D2 backgrounds exhibited less anxiety-like behavior ($p = 0.004$ and $p = 0.030$).

The light-dark exploration test was developed and validated by Crawley and Goodwin (1980). The number of transitions between the light and dark compartments is sensitive to treatment with anxiolytics. There was an overall effect of genotype ($F(1, 175) = 3.929, p = 0.049$) in which *Fmr1*KO mice made more transitions (Figure 3B). Looking at the individual lines, only the *Fmr1*KO mice on the congenic B6 background made significantly more transitions than WT littermates indicating they displayed less anxiety-like behavior ($p = 0.027$). Some backgrounds make very few transitions and spend most of the time in the dark compartment, so risk assessment behaviors were also analyzed (data not shown). Despite making very few transitions, B6A-*Fmr1*KO mice made more stretch-attend postures from the dark compartment into the light compartment, suggesting less anxiety-like behavior than their WT littermates ($p = 0.002$).

Mice were given novel food while they were housed individually in preparation for testing for social behavior (Spencer *et al.*, 2008). Mice do not typically consume very much of a novel food, an anxiety-related response called ‘hypo-neophagia’. An overall main effect of genotype was observed ($F(1, 175) = 17.545, p < 0.0005$) in which *Fmr1*KO mice consumed more of the blueberry bar than their WT littermates (Figure 3C). This difference was significant in four of the backgrounds: congenic B6 ($p = 0.001$), B6A ($p = 0.042$), B6D2 ($p = 0.005$) and B6S1 ($p = 0.050$).

Depression is not a primary characteristic of FXS, although individuals may be at increased risk secondary to other problems considered part of the FXS phenotype (Sobesky *et al.*, 1995). Depression-related behavior in the mice was examined in a tail suspension test. No overall main effect of genotype was observed ($F(1, 174) = 1.111, p = 0.293$). There was however a trend in which *Fmr1*KO mice on the B6CD background spent less time immobile than their WT littermates ($p = 0.064$; data not shown).

Sensory responses/sensorimotor gating and adaptation

Auditory response and sensorimotor gating were assessed by prepulse inhibition of the acoustic startle response. In normal animals the startle response to a loud 120 dB sound stimulus is reduced when the startle stimulus is immediately preceded by a weaker non-startling stimulus (prepulse). Individuals with FXS exhibit abnormal acoustic startle responses and PPI (Frankland *et al.*, 2004).

For the acoustic startle response (Figure 4A), there was a main effect of genotype ($F(1, 175) = 16.834, p < 0.0005$) in which *Fmr1*KO mice showed a diminished startle response to the 120 dB startle stimulus compared to WT animals. A reduced acoustic startle response was observed in the B6A ($p = 0.015$) and B6S1 ($p = 0.001$) backgrounds specifically. There was a trend for *Fmr1*KO mice on the congenic B6 background ($p = 0.101$). For prepulse inhibition of the acoustic startle response (Figure 4B), there was an overall main effect of genotype ($F(1, 175) = 7.803, p = 0.006$) with *Fmr1*KO mice showing enhanced PPI relative to WT littermates. Looking at specific backgrounds, this result was observed in the congenic B6 ($p = 0.002$) and B6F ($p = 0.040$) lines.

A day following the test for prepulse inhibition, mice were returned to the apparatus for assessment of acoustic startle habituation, in which the 120 dB startle stimulus was presented 100 times. Repeated measures ANOVA indicated no main effect of genotype across all the lines ($F(9, 1575) = 1.013, p=0.427$) and no effect within any of the specific backgrounds ($p's > 0.05$), indicating no difference between *Fmr1*KO and WT mice in acoustic startle habituation (data not shown).

Many individuals with FXS exhibit self-injurious behaviors such as hand-biting (Symons *et al.*, 2003), suggesting that they may be less sensitive to pain. Mice were tested for analgesia-related responses to a 55°C hotplate. There was a main effect of genotype ($F(1, 159) = 12.274, p= 0.001$) with *Fmr1*KO mice showing an increased latency to show a hindlimb response (Figure 4C). This response was significant in the B6D2 line ($p=0.052$) and trends were observed in the congenic B6 ($p=0.059$), B6A ($p=0.058$) and B6F ($p=0.083$) lines.

Learning and memory

Approximately 87% of males with full FXS mutations have cognitive impairment, defined as an IQ less than 70 (Hagerman, 2002). Cognitive impairment in the mice was tested using fear conditioning, a learning and memory paradigm in which mice were trained to associate contextual and sound cues with foot shock by freezing in response to the cues presented separately 24 h after training. In the context test, there was no main effect of genotype (Figure 5A; $F(1, 175) = 0.353, p= 0.553$). However, B6CD-*Fmr1*KO mice were significantly impaired relative to their WT littermates ($p=0.029$). In the (sound) cued test, there was no main effect of genotype (Figure 5B; $F(1, 175) = 0.011, p= 0.917$) and there were no significant differences between *Fmr1*KO and WT mice in any of the backgrounds tested.

Social behavior

An estimated 61–73% of males with FXS exhibit social avoidance or withdrawal (Hagerman, 2002). Furthermore, difficulty with social interaction is a core diagnostic criterion for autism. Mice were evaluated for social interest and recognition in the partition test and a direct social interaction test. All subjects were partnered with standard age- and weight-matched C57BL/6J mice.

Partition test for social recognition and interest

The social partition test (Kudryavtseva, 2003; Spencer *et al.*, 2005) measured social interest and recognition in a familiar environment during three 5 min encounters across a partition with an overnight “familiar” partner first and a novel partner second. The original overnight partner was returned for the third test (data not shown) to confirm the recognition ability of the subject. *Fmr1*KO and WT mice in all strains showed normal social recognition, which was indicated by a significant increase in time spent at the partition during the second test with the novel partner followed by a significant decrease in the time at the partition during the third test when the original partner was returned ($p's < 0.05$).

There was no overall effect of the *Fmr1* null mutation in time spent at the partition during the first, or baseline, test with the overnight ‘familiar’ partner (Figure 6A; $F(1, 174) = 0.587, p= 0.445$). However, *Fmr1*KO mice on the B6S1 background spent significantly more time at the partition than their WT littermates ($p=0.019$). There was also no overall genotype effect in time spent at the partition (normalized to the baseline test) during the second test with a novel partner (Figure 6B; $F(1, 174) = 0.000, p= 0.992$) nor during the third test when the original partner was returned (data not shown; $F(1, 174)= 0.129; p= 0.720$). B6S1-*Fmr1*KO mice however spent significantly less time than WT littermates at the partition when presented with a novel partner ($p=0.023$). Thus, in the partition test, a genotypic

difference in the level of social interest was evident in only one of the six genetic backgrounds tested, while no differences were observed in social recognition. Similar to our previous study with mice with no prior test history (Spencer *et al.*, 2005), *Fmr1*KO mice on the congenic B6 background did not show any differences in the partition test.

Direct social interaction

After the partition test, mice were housed in the partitioned cages for 2 additional days with their original overnight partners and then given a 10 min direct social interaction test. There was no overall genotype effect for time spent in active social behavior initiated by the subject (Figure 7A; $F(1, 174) = 0.387$, $p = 0.535$). *Fmr1*KO mice on the congenic B6 background showed increased active social behavior ($p = 0.003$) while *Fmr1*KO mice on the B6D2 background showed a decrease in active social behavior ($p = 0.05$). Since active social behavior could be either investigative or aggressive in nature, we also looked at these subtypes of behavior (data not shown). An overall effect of the *Fmr1* mutation was observed in investigative active social behavior ($F(1, 174) = 6.001$; $p = 0.015$). *Fmr1*KO mice on the congenic B6 and B6F backgrounds showed significantly increased levels of investigative social behavior ($p < 0.005$ and $p = 0.030$, respectively). There was no main effect of genotype overall nor in any of the six lines for aggressive active social behavior. *Fmr1*KO mice on the B6D2 background were not significantly different from WT littermates in either investigative ($p = 0.127$) or aggressive ($p = 0.120$) active social behavior; thus the decrease in overall active social behavior was general in nature.

Passive social behavior was defined as the behavior of the subject responding to behavior initiated by the standard partner mouse. Several different responses were possible, ranging from receptive to fleeing or freezing. An overall effect of the *Fmr1* mutation was observed in passive/receptive social behavior (Figure 7B; $F(1, 174) = 5.175$, $p = 0.024$) with *Fmr1*KO mice showing an overall decrease. Looking at individual backgrounds, there was a decrease in passive/receptive social behavior in B6S1-*Fmr1*KO mice ($p = 0.002$) and trend toward a decrease in congenic B6-*Fmr1*KO mice ($p = 0.083$).

There was also a trend toward an overall genotype effect in time spent in nonsocial behavior (Figure 7C; $F(1, 174) = 2.754$, $p = 0.099$), although a significant difference was found only in B6S1-*Fmr1*KO mice which showed an increase in nonsocial behavior ($p = 0.013$). B6D2-*Fmr1*KO mice showed a trend toward increased nonsocial behavior ($p = 0.084$) and B6F-*Fmr1*KO mice showed a trend toward decreased nonsocial behavior ($p = 0.094$).

Since B6D2-*Fmr1*KO mice had displayed increased stereotypic behavior in the open field and marble bury tests (Figures 3A and 3B) and also increased activity and rearing in the open field (Figures 1A and 1B), we were interested in determining whether an increase in these behaviors could explain the decrease in active social behavior in these mice. However, there was no significant difference between B6D2-*Fmr1*KO and WT mice in any of the behavioral subgroups within the category “non-social behavior”, i.e. exploratory activity, rearing, digging, self-grooming, or rest behaviors (p 's > 0.05 ; data not shown). Thus the decreased active social behavior in B6D2-*Fmr1*KO mice cannot be explained by a preoccupation with stereotypic behaviors or hyperactivity.

Ultrasonic vocalizations in B6D2-*Fmr1* neonatal mice in response to isolation

After the initial test battery, we saw that the *Fmr1*KO mice on the B6D2 background had a behavioral phenotype that showed two out of three of the core features of autism, namely impaired social interaction and repetitive behavior. The third core feature of autism is impaired social communication, which in mice has been looked at by analyzing their ultrasonic vocalizations (Branchi *et al.*, 2001; Scattoni *et al.*, 2009). Mouse pups will

vocalize at frequencies between 30 and 90 kHz in response to being separated from their dam and littermates (Branchi *et al.*, 2001). Isolation-induced USVs were measured in pups of B6D2 background from postnatal days (PND) 3–14. The number of calls made by male *Fmr1*KO and WT pups over this timecourse is shown in Figure 8. There was a main effect of genotype with male *Fmr1*KO mice making more calls than their male WT littermates ($F(1, 36) = 6.919, p = 0.012$).

Discussion

A summary of the results is presented in Table 1. An overall main effect of genetic background was observed in almost all measures generated (both reported and not reported). This indicates that the genetic differences generated in this study were sufficient to generate significant effects on the behavioral parameters. Even with significant background differences, there were several overall differences between *Fmr1*KO and WT mice across all backgrounds:

- *Fmr1*KO mice were more active in the open field.
- *Fmr1*KO mice showed less anxiety-like behavior in the open field and light-dark chambers and toward novel food.
- *Fmr1*KO mice exhibited decreased acoustic startle response and enhanced PPI.
- *Fmr1*KO mice were less sensitive to hotplate.
- *Fmr1*KO mice showed increased investigative social behavior, decreased passive/receptive social behavior and a trend for increased non-social behavior in the social interaction test.

The overall picture that emerges is that the *Fmr1*KO mouse is hyperactive, less anxious (potentially confounded by activity), and has abnormal sensory and social responses. With the exception of the hotplate response, which was not previously reported, many of these behaviors were observed previously in *Fmr1*KO mice on the congenic B6 background and have been shown to be (over)corrected in *Fmr1*KO mice expressing transgenic FMRP (Peier *et al.*, 2000; Paylor *et al.*, 2008; Spencer *et al.*, 2008). Decreased sensitivity to the hotplate was expected based upon the self-injurious behavior of many individuals with FXS. Furthermore, it has previously been reported that *Fmr1*KO mice on the congenic B6 background show reduced spinal and peripheral nociceptive sensitization (Price *et al.*, 2007). Some of these phenotypes (anxiety-related behavior, PPI) were contrary to the expected results based upon the human phenotype. These mouse-human behavioral differences have been observed and discussed in previous studies (Peier *et al.*, 2000; Spencer *et al.*, 2005; Paylor *et al.*, 2008). For many of these phenotypes, sometimes the effect size was greater in particular backgrounds, perhaps due to different baseline responses in the F1 WT mice (see Owen *et al.*, 1997, and Logue *et al.*, 1997 for presentation of differences among F1 lines), indicating that one could select backgrounds with more robust differences to improve experimentation.

In a few of the backgrounds, additional behavioral phenotypes were observed:

- *Fmr1*KO on the B6CD background were impaired in contextual fear learning.
- *Fmr1*KO mice on the B6F background showed reduced habituation to novel environment (open field).
- *Fmr1*KO mice on the B6S1 background showed abnormal social responses in the partition test as well as in the social interaction test. The enhanced interest in

familiar partners combined with the reduced interest in unfamiliar partners suggests a social anxiety-like phenotype.

- *Fmr1*KO mice on the B6D2 background showed increased repetitive behavior in open field and marble bury tests, and reduced active social behavior in the social interaction test. In addition, *Fmr1*KO mice on the B6D2 background showed increased USVs in response to isolation during the early postnatal period.

Many of these additional behavioral phenotypes in the mouse models are consistent with behaviors observed in individuals with FXS, such as intellectual disability (approximately 87%), repetitive or stereotypic behaviors (85–100%), and abnormal social behavior (61–73%) (Hagerman, 2002). The finding of a contextual fear phenotype in the B6CD background is of particular interest given the difficulty in detecting learning and memory phenotypes in the *Fmr1*KO mouse model (see discussion in Spencer *et al.*, 2006). We previously observed a contextual fear impairment and a decreased habituation phenotype in *Fmr1/Fxr2* double KO mice (Spencer *et al.*, 2006), which suggested that *Fxr2* was compensating for the loss of *Fmr1* in these behavioral responses. The results of the present study suggest that the CD-1 and FVB backgrounds contribute modifier genes that over-ride compensatory mechanisms present in the B6 background. A social anxiety-like phenotype was observed previously in the congenic B6 background under special experimental conditions (Spencer *et al.*, 2005); however the phenotype appears more pronounced on the B6S1 background. Differences in exploratory activity might be involved as mice on the B6S1 background are much less active than B6 mice.

Our usual battery of behavioral tests was expanded to include tests for repetitive behavior (marble bury) and social behavior in order to screen for autism-related phenotypes. After a particular line (B6D2) was identified from the initial battery to be of potential interest, it was further tested for a social communication phenotype. Given a behavioral profile that touches on all three of the core features of autism, the *Fmr1*KO mice on the B6D2 genetic background in particular look promising as a model for FXS with autism.

We would be remiss to not comment on the fact that although there are a number of behavioral phenotypes in the various lines of *Fmr1*KO mice, that for most responses, there were no significant differences between KO and WT mice in many of the backgrounds. This observation clearly demonstrates that genetic background has a major impact on the presentation of the *Fmr1* phenotype, and one could argue that the genetic background more than the mutation determines the final phenotypic pattern.

Autism-related behaviors in *Fmr1*KO mice

The core features of autism are impaired social interaction, impaired social communication and the presence of perseverative or repetitive behaviors or restrictive interests. Considerable debate exists in defining how these human behaviors should be represented in a valid mouse model of autism. Many researchers suggest that the mouse is a good model if they exhibit only one of the core behaviors associated with autism. However, while almost all males with FXS exhibit at least one of these core symptoms of autism, only 21–50% meet the full diagnostic criteria for autism (Moss & Howlin, 2009). Thus, the question becomes whether all valid mouse behavioral models of FXS should be considered good mouse models of autism.

Of all the core autism-related behavioral domains, social interaction has been most closely looked at in *Fmr1*KO mice. Previous reports have described the presence of abnormal social behavior in the *Fmr1*KO mice on a congenic C57BL/6 background (Spencer *et al.*, 2005; Mineur *et al.*, 2006; Spencer *et al.*, 2008; Mines *et al.*, 2010), a congenic FVB background (Liu & Smith, 2009; Moy *et al.*, 2009), and on a B6 × FVB hybrid background

(McNaughton *et al.*, 2008). The majority of these studies suggested elevated levels of social anxiety in the presence of unfamiliar partners (Spencer *et al.*, 2005; McNaughton *et al.*, 2008; Liu & Smith, 2009; Moy *et al.*, 2009; Mines *et al.*, 2010). When presented with a very familiar partner, however, *Fmr1*KO mice respond with increased social approach, reflected mostly by increased sniffing of the partner (Spencer *et al.*, 2005; Spencer *et al.*, 2008). Importantly, this behavior was corrected by transgenic expression of FMRP in the knockout mouse, indicating that FMRP levels contribute to this social phenotype (Spencer *et al.*, 2008). This finding was also replicated in this study as the *Fmr1*KO mice on the congenic B6 background exhibited increased active social behavior in the social interaction test. Although consistent with a FXS-like phenotype (see discussion in Spencer *et al.*, 2005), the presence of increased social approach with familiar partners is not necessarily consistent with an autism-like phenotype. The increased investigative social behavior of *Fmr1*KO mice on the congenic B6 and B6F backgrounds is perhaps related to their increased exploratory activity since *Fmr1*KO mice on these backgrounds were hyperactive in the open field. Interestingly, although B6D2-*Fmr1*KO mice also explored the open field more actively than their WT littermates, they showed decreased overall active social behavior with no difference in investigative social behavior.

The second core feature of autism is impaired social communication. In mice, social communication has routinely been assessed by measuring ultrasonic vocalization (USV), especially isolation-induced vocalization in neonatal mice (Branchi *et al.*, 2001; Scattoni *et al.*, 2009). To our knowledge, the experiment evaluating pup USVs in the B6D2-*Fmr1* mice is the first published study of USVs in any *Fmr1* mouse model. As a first attempt to assess social communication in these mice, this experiment was performed only in the background (B6D2) that was most promising as a potential model for autistic-like traits. Many mouse models of autism-related neurodevelopmental disorders have been analyzed in this manner and alterations in the total number of 'calls' at particular ages or the developmental trajectory have been observed in the majority of these models (reviewed in Moy & Nadler, 2008). It is logical that most investigators would have predicted *a priori* that pups would vocalize less in response to isolation since humans with autism spectrum disorders usually are less communicative (Crawley, 2004). However, while some of the genetic mouse models associated with autism, such as neuroligin-3 (Radyushkin *et al.*, 2009) and neuroligin-4 (Jamain *et al.*, 2008) do show decreased USVs, other genetic models such as the *Mecp2* null Rett syndrome mouse (Picker *et al.*, 2006) and the 15q11-13 duplication mouse (Nakatani *et al.*, 2009) exhibited increased calling during the neonatal period. The inconsistency of these results together with inconsistent reports regarding the amount of crying in human infants who are later diagnosed with autism, has led some to question whether isolation-induced vocalizations in rodent pups are relevant to social communication in autism.

Infant cries are the earliest form of social communication, intended to elicit caregiver attention. Similar to human infants, the calls of neonatal mice elicit retrieval behavior in the parents (Ehret, 2005), thus there is face validity for studying this behavior as a model for human infant social communication. The natural process of call followed by response can be altered by abnormal calling by the infant or by abnormal response of the parent. Furthermore, maternal response can feedback to modulate infant calling; in mice there is a correlation between maternal responsiveness and the number of isolation-induced pup USVs such that pups that receive less maternal care call more than pups that receive more maternal care (D'Amato *et al.*, 2005). In the case of the B6D2-*Fmr1*KO mice, there was an increased number of calls as compared to their WT littermates, a finding which is similar to other genetic mouse models of autism mentioned above but counter to what may have been expected *a priori* for an autism-related phenotype. We suggest that the increased calling in the B6D2-*Fmr1*KO neonatal mice and possibly other mouse models of autism could be a reflection of less maternal care, which could reflect the quality of the pups' communication.

The quality of human infant cries could be an important early characteristic of autism. Analysis of retrospective videotapes of infants with autism has revealed that the acoustic properties of their cries are different from the cries of typically-developing infants and their cries are considered more aversive and distressing to adult caregivers (Esposito & Venuti, 2010). Thus further studies in the B6D2-*Fmr1*KO mouse model will be important to analyze the detailed characteristics of the vocalization patterns, such as performed for BTBR mice (Scattoni *et al.*, 2008), and also to assess whether there are any differences in the maternal responses to *Fmr1*KO compared to WT offspring. Future studies of vocalizations during adult social interaction will also be informative, since communication difficulties persist throughout life for individuals with autism.

Perseveration, resistance to change, or stereotypic/repetitive behavior is another core feature of autism. Children with autism often show both repetitive motor mannerisms and cognitive rigidity (Carcani-Rathwell *et al.*, 2006). In mice, these behaviors have been modeled in two different ways. One approach has been to look at reversal learning paradigms such as Morris water maze or appetitive t-maze (Crawley, 2007; Moy *et al.*, 2007; Moy *et al.*, 2008). In early reports about *Fmr1*KO mice on a mixed genetic background, impairments in reversal learning were noted in the Morris water maze assay (Bakker *et al.*, 1994; Kooy *et al.*, 1996; D'Hooze *et al.*, 1997). Some investigators suggested that this is an example of resistance to change that supports the *Fmr1*KO mouse model as a model for autism (Bernardet & Crusio, 2006; Moy *et al.*, 2006). However, it is difficult in the Morris water maze to ensure that the mice are at equivalent levels in the initial test before initiating the reversal trials. This is especially a concern when there are reports of learning tasks in which *Fmr1*KO mice performed better than their WT littermates (Fisch *et al.*, 1999; Frankland *et al.*, 2004). Mice that are overtrained relative to their controls would be expected to reverse more slowly. Furthermore, swim latencies were used as the primary evidence for impaired reversal, and swim latencies can be influenced by many factors that are not specific to learning. For example, *Fmr1*KO mice have been reported to have mild motor learning impairments (Peier *et al.*, 2000) and in one water maze experiment it was noted that the *Fmr1*KO mice did not swim as fast in later trials (D'Hooze *et al.*, 1997). Thus previous reports of impaired reversal learning in the Morris water maze are not particularly convincing with respect to a perseverative behavioral phenotype in *Fmr1*KO mice. We suggest that clearer conclusions would be obtained by using assays such as a t-maze task that has defined indicators of learning such as trials to criterion (Crawley, 2007; Moy *et al.*, 2007; Moy *et al.*, 2008). One assay we recommend is a simple perseveration assay in a water t-maze in which mice are trained to go either left or right to an escape platform. This task takes advantage of a behavior in which mice naturally persevere. For each individual mouse, after achieving 9 out of 10 correct trials for 2 consecutive days, the platform is moved to the opposite location and the number of trials to reach a criterion of 9 out of 10 correct trials in the new location is determined. We have performed this experiment with *Fmr1*KO mice on the congenic B6 background and did not find evidence of perseveration. No difference was observed between *Fmr1*KO and WT littermates in the number of trials to criterion for the initial task to find the escape platform in one arm nor for the reversal task in which the escape platform was moved to the other arm nor for a second reversal trial (C. Spencer, unpublished results). We believe that behavioral tasks such as this may be more appropriate to test for perseverative or inflexible learning behavior in mice.

Another approach to determining if mice are resistant to change is to look directly at the reaction of mice when confronted with a reversal learning task. Moon and colleagues (2008) looked at the behaviors of *Fmr1*KO mice on a B6 × FVB hybrid background as they were confronted with changing task demands during olfactory discrimination learning. Although there was no difference in measures of learning during acquisition and reversal, *Fmr1*KO mice showed increased activity and wall-climbing when contingencies were changed in the

reversal learning task and also in trials that followed an error. In another study using the five-choice visual attention task, these authors found that *Fmr1*KO mice were more impulsive (making more premature responses), had difficulty maintaining attention, and exhibited increased arousal (more wall-climbing) after task changes or errors (Moon *et al.*, 2006). These studies offer compelling evidence that *Fmr1*KO mice exhibit heightened emotional responses when confronted with changes during learning paradigms.

Repetitive or stereotypic behaviors in mice can also be studied by observing the mice in their home-cage or in novel environments. Thus it is a challenge to detect repetitive behavior in otherwise normal-looking mice. Indeed, to our knowledge, there have been no published reports of repetitive behavior in *Fmr1*KO mice until this study. Our laboratory recently reported that the marble bury assay reflects repetitive digging behavior (Thomas *et al.*, 2009). Although marble bury was originally identified as a test for anxiety-like responses (Broekkamp *et al.*, 1986; Njung'e & Handley, 1991), this activity did not correlate with anxiety-related measures in the open field test or with light-dark, but instead with digging behavior and the stereotypy measure in the open field test (Thomas *et al.*, 2009). Thus in the present study, we included both stereotypy measures from the open field test and the marble bury test in our comprehensive test battery. Holeboard exploration was recently recommended as a test for perseverative or repetitive behavior (Moy *et al.*, 2008), and we have observed that *Fmr1*KO mice on the congenic B6 background show more repetitive behavior than WT littermates in this assay (C. Spencer, unpublished results). Thus we think the holeboard assay will be useful in further screening and characterizing repetitive behavior in *Fmr1*KO mice.

Fmr1KO mice on the B6D2 F1 background - a model of FXS with autism

We believe that the optimal mouse model of FXS with autism would be one in which the mouse equivalents of all three core features of autism are present. In that regard, the *Fmr1*KO mouse on the B6 × DBA (B6D2) hybrid background satisfies all three of these behavioral requirements and warrants further investigation. This mouse model has increased repetitive behavior as observed in the open field and marble bury tests. Also, this mouse model exhibits a social behavior abnormality that is different from other *Fmr1*KO models but more consistent with autism-like social behavior in that they show decreased active social approach behavior during social interaction with a familiar partner. Furthermore, *Fmr1*KO mice on this background have abnormal isolation-induced vocalizations during the neonatal period. We regard these behavioral phenotypes of B6D2-*Fmr1*KO mice to minimally satisfy the core requirements for a mouse model of autism; further characterization and validation of the model will be important and necessary.

The current findings, together with previously published reports, clearly demonstrate the importance of genetic background on behavioral responses in the *Fmr1*KO mice. Based on our results, one might even suggest that the genetic background is the determining factor defining the nature of the phenotype in *Fmr1*KO mice. Our findings also indicate that variability between individuals in FXS is likely due in part to other background genes. One point that should not be overlooked is that by examining six distinct, but related, genetic backgrounds and observing behavioral differences on every background, we have shown that the interaction between the *Fmr1* mutation and other genes models the variability observed in FXS. Lastly, the results indicate that the B6D2-F1 *Fmr1*KO mice display behavioral abnormalities consistent with the three domains of autism spectrum disorders (ASD), suggesting that this line may be an optimized *Fmr1*KO line for understanding the role of *FMR1* in ASD. As a result of our breeding strategy, the behavioral differences between *Fmr1*KO and WT responses in the B6A, B6D2, B6F, or B6S1 F1 hybrid lines compared to *Fmr1*KO and WT responses in the congenic B6 line are presumably due to dominant modifier effects of genes contributed by the non-B6 strains. Thus it should be

informative to perform genetic screens and determine which genes from the DBA strain are modifying the behavior of *Fmr1*KO mice to produce the autism-related phenotypes in the B6D2 line. This could lead to a greater understanding of the biological mechanisms involved in autism spectrum disorders and lead to new therapeutic interventions.

Acknowledgments

This research was supported by the Administrative and Neurobehavioral Cores of the NIH/NICHD Baylor Intellectual and Developmental Disabilities Research Center and the Baylor Fragile X Research Center.

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Figure 1.

Exploratory activity in the open field for *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds. Bars represent means $\pm$ SEM for total distance travelled in centimeters (cm) (A), and for vertical activity (B). Data are averages of two 30 min tests on consecutive days. Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

Figure 2. Repetitive behavior in *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds was assessed by stereotypy counts in the open field (A) and number of marbles buried in the marble bury test (B). Bars represent means $\pm$ SEM. Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

Figure 3. Anxiety-related behaviors in *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds. Bars represent means $\pm$ SEM for center distance: total distance ratio in the open field test (A), number of transitions in the light-dark test (B), and amount of novel blueberry bar consumed in 20 min (C). Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

Figure 4. Sensory responses and sensorimotor gating in *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds. Bars represent means $\pm$ SEM for maximum response to 120 dB acoustic startle (A), average percent prepulse inhibition to low level (74–82 dB) prepulse sound levels (B), and latency to exhibit a hind limb response in the hotplate test (C). Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

A. Context Test

B. Cued Test

Figure 5.

Fear conditioning in *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds. Bars represent means $\pm$ SEM for percent freezing in (A) contextual fear test and (B) cued fear test. Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

A. Familiar Partner

B. Unfamiliar Partner

Figure 6.

Social behavior in the partition test for *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds. Bars represent means $\pm$ SEM for time in sec at the partition during the baseline test with familiar overnight partner (A) and percent time at the partition with the unfamiliar partner relative to the baseline time (B). Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

Figure 7.

Social behavior in a social interaction test. *Fmr1*KO (black bars) and WT littermates (white bars) in six genetic backgrounds spent 10 min interacting with C57BL/6J partner mice after 48 hr in partitioned housing. Bars represent means $\pm$ SEM for percentage of time spent in active social behavior (A), passive/receptive social behavior (B) and nonsocial behavior (C). Asterisks indicate a significant difference ($p \leq 0.05$) between *Fmr1*KO and WT littermates within a specific line.

Figure 8.

Isolation-induced ultrasonic vocalizations in male neonatal *Fmr1*KO mice (closed circles) and WT littermates (open circles) in the B6D2 genetic background. Circles represent means $\pm$ SEM for the number of calls recorded each day from postnatal day 3 to 14 during a 2 min isolation period from dam and littermates.

Table 1

Behavioral differences in *Fmr1*/KO versus WT mice on six genetic backgrounds.

Type of Behavior	Test	Parameter	B6	B6A	B6D2	B6F	B6S1	B6CD	effect of <i>Fmr1</i>	all KO	strain
Locomotor/exploratory	Open Field	Total distance travelled Vertical Activity	↑	=	↑	↑	=	=	p<0.001 p=0.036	↑ ↓	p<0.001 p<0.001
			=	=	↑	=	↓	↓			
Motor coordination and skill learning	Rotarod	Time walking on rotating rod	=	=	=	=	↑	=	n.s.	=	p=0.002
Repetitive behavior/stereotypy	Open Field Marble Bury	Stereotypy (STACT) No. of marbles buried	=	=	↑	=	=	=	p=0.009 n.s.	↑ =	p<0.001 p<0.001
Anxiety-related behavior	Open Field Light-Dark Novel Taste	Center distance ratio Light-Dark transitions Amount of food eaten	=	↑	↑	=	=	=	p=0.005 p=0.049 p<0.001	↑ ↑ ↑	p<0.001 p<0.001 p=0.018
Depression-related behavior	Tail Suspension	Duration, immobility	=	=	=	=	=	(↓)	n.s.	=	p<0.001
Sensory responses	Acoustic Startle Hotplate	Acoustic startle response Latency to first response	(↓)	↓	=	=	↓	=	p<0.001 p=0.001	↓ ↑	p<0.001 p<0.001
			(↑)	(↑)	↑	(↑)	=	=			
Sensorimotor gating and adaptation	Acoustic Startle	Pre-pulse inhibition Startle habituation	↑	=	=	↑	=	=	p=0.006 n.s.	↑ =	p<0.001 p<0.001
Learning and memory	Conditioned Fear	Contextual fear conditioning Cued fear conditioning	=	=	=	=	=	↓	n.s. n.s.	= =	p<0.001 p=0.017
Social behavior	Partition Test	Social interest, familiar partner	=	=	=	=	↑	=	n.s.	=	p<0.001
		Social interest, unfamiliar partner	=	=	=	=	↓	=	n.s.	=	n.s.
	Social Interaction	Active social behaviors	↑	=	↓	=	=	=	n.s.	=	p<0.001
		Active: investigative	↑	=	=	↑	=	=	p=0.015	↑	p<0.001
		Passive social behaviors	(↓)	=	=	=	↓	=	p=0.024	↓	n.s.
		Nonsocial behaviors	=	=	(↑)	(↓)	↑	=	p=0.099	(↑)	p<0.001

=, no difference between KO and WT mice, $p > 0.10$

↑, KO>WT, $p \leq 0.05$; (↑), trend KO>WT, $0.05 < p \leq 0.10$

↓, KO<WT, $p \leq 0.05$; (↓), trend KO<WT, $0.05 < p \leq 0.10$