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Comprehensive Motor Testing in *Fmr1*-KO Mice Exposes Temporal Defects in Oromotor Coordination

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Abstract

Fragile X syndrome (FXS; MIM #300624), a well-recognized form of inherited human mental retardation is caused, in most cases, by a CGG trinucleotide repeat expansion in the 5'-untranslated region of *FMR1*, resulting in reduced expression of the fragile X mental retardation protein (FMRP). Clinical features include macroorchidism, anxiety, mental retardation, motor coordination, and speech articulation deficits. The *Fmr1* knockout (*Fmr1*-KO) mouse, a mouse model for FXS, has been shown to replicate the macroorchidism, cognitive deficits, and neuroanatomical abnormalities found in human FXS. Here we asked whether *Fmr1*-KO mice also display appendicular and oromotor deficits comparable to the ataxia and dysarthric speech seen in FXS patients. We employed standard motor tests for balance and appendicular motor coordination, and used a novel long-term fluid-licking assay to investigate oromotor function in *Fmr1*-KO mice and their wild-type (WT) littermates. *Fmr1*-KO mice performed equally well as their WT littermates on standard motor tests, with the exception of a raised-beam task. However, *Fmr1*-KO mice had a significantly slower licking rhythm than their WT littermates. Deficits in rhythmic fluid-licking in *Fmr1*-KO mice have been linked to cerebellar pathologies. It is believed that balance and motor coordination deficits in FXS patients are caused by cerebellar neuropathologies. The neuronal bases of speech articulation deficits in FXS patients are currently unknown. It is yet to be established whether similar neuronal circuits control rhythmic fluid-licking pattern in mice and speech articulation movement in humans.

Keywords

oromotor behavior; fragile X syndrome; motor coordination; motor learning

Fragile X syndrome (FXS) is the most common form of inherited mental retardation in humans. Prevalence estimates range from 1 in 1200 to 1 in 2500 males, and from 1 in 1600 to 1 in 5000 females (Hagerman & Hagerman, 2002). In approximately 95% of all cases, the syndrome is caused by an expanded CGG trinucleotide repeat in the 5'-untranslated region

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of the *FMR1* gene, resulting in abnormal promoter methylation and loss of gene expression (Fu et al., 1991; Oberle et al., 1991). Loss of *FMR1* expression has been reproduced in a widely used mouse model of FXS, the *Fmr1*-KO mouse ("Fmr1 Knockout Mice," 1994). The *FMR1* gene is expressed ubiquitously throughout the brain (Abitbol et al., 1993; Devys, Lutz, Rouyer, Bellocq, & Mandel, 1993; Pacey & Doering, 2007). In neurons, fragile X mental retardation protein (FMRP) localizes to the somata, dendrites, and within dendritic spines (Antar, Afroz, Dictenberg, Carroll, & Bassell, 2004; Feng et al., 1997; Weiler et al., 1997). Spines in neocortical pyramidal cells, cerebellar Purkinje cells, and other types of neurons are increased in number, abnormally thin, and elongated in human FXS patients and the *Fmr1*-KO mouse (Comery et al., 1997; Fiala, Spacek, & Harris, 2002; Koekkoek et al., 2005). FMRP is an mRNA binding protein involved in translational suppression (Jin & Warren, 2003). Thus, even though FXS is a single gene disorder, it is likely to cause the abnormal regulation of many genes. FXS is characterized by cognitive impairments, developmental delay, anxiety, and increased risk for autism spectrum disorder (Hagerman, Ono, & Hagerman, 2005; Todd & Malter, 2002), as well as motor coordination and speech articulation deficits (Dobkin et al., 2000; Fiala et al., 2002; Koekkoek et al., 2005; Paradee et al., 1999; Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004). Here we asked whether the *Fmr1* deficient mouse model of FXS displays motor coordination and oromotor deficits. To test oromotor function, we employed a long-term fluid-licking assay (Heck, Zhao, Roy, LeDoux, & Reiter, 2008). We used a battery of standard motor tests to evaluate balance, motor coordination, and motor learning.

Method

Breeding pairs of FVB.129P2-*Fmr1*^{tm1Cgr/J} mice were purchased from the Jackson Laboratory (Bar Harbor, ME stock # 004624) and bred by pairing wild-type males with heterozygous females in a conventional mouse vivarium at the University of Tennessee Health Science Center using harem breeding trios. Pups were kept with the dam until weaning at Postnatal Day 21 (P21). Juveniles were housed by gender in standard plastic cages not exceeding four per cage. All mice used in this study were raised, and all experiments performed, in accordance with procedural guidelines approved by the University of Tennessee Health Science Center Animal Care and Use Committee. Principles of laboratory animal care (National Institutes of Health, 1986) were followed. Only male WT and *Fmr1*-KO littermates were used for testing to avoid variability in behavioral performance because of mosaicism of the mutant allele due to X-inactivation or the estrous cycle in females. All behavioral experiments were performed with 7- to 15-week-old mice (mean age = 11.994, *SE* = 0.392). All mice were weighed before behavioral testing began, and the measurements were used for statistical comparison of body weights between *Fmr1*-KO and WT mice. Behavioral testing was performed by investigators blinded to phenotype. Rotarod performance, the elevated beam task, footprint analysis, the vertical rope-climbing assay, and grip strength were examined during afternoon sessions (1 to 5 p.m.), held constant across mice. After completion of behavioral tests, tails were collected from the animals and processed for genotyping (Transnetyx, Inc., Cordova, TN).

Long-Term Licking Behavioral Assay

Male *Fmr1*-KO mice ($N = 14$) and their WT littermates ($N = 14$) were singly housed in their home cage environment while spontaneous water-licking behavior was electronically monitored for an uninterrupted 72-h period, as previously described (Heck et al., 2008). The normal 12-hr light–dark cycle continued as before (light: 6 a.m. to 6 p.m.). Dark and light periods were registered in the same data file as licking data, in the form of the voltage output from a photovoltaic cell. Mice had unrestricted access to food and water during the entire test period. The cages were modified to detect contact between the mouse's tongue and the waterspout based on the junction potentials (Hayar, Bryant, Boughter, & Heck, 2006). The modifications included the placement of a metal wire mesh on the floor underneath the waterspout to serve as an electrical reference. The size of the wire mesh (10×15 cm) was sufficient to ensure that the mouse had to stand on the mesh in order to access the waterspout. The mesh and waterspout were connected to ground and the signal input of a BNC connector socket of an analogue to digital converter (CED 1401; Cambridge Electronic Design, Cambridge, UK). During fluid licking, each tongue-to-waterspout contact resulted in a junction potential of 0.1- to 1-V amplitude that would last as long as the tongue-to-waterspout contact continued. The junction potential signals were digitized at 1200 to 2000 Hz and stored on hard disk. Lick events were analyzed offline using *Spike2* software (Cambridge Electronic Design). The quantitative parameters were extracted from the licking data as follows. First, the mean duration of the interlick interval (ILI) was used as a measure for the speed of the lick rhythm. Only ILI durations ranging from 40 to 250 ms were included in the ILI statistical analysis in order to omit pauses between lick bursts and intervals between visits. Second, the coefficient of variation of the ILI distribution (again limited to intervals between 40 and 250 ms) was used as a measure for the variability of the lick rhythm. Third, for a more comprehensive statistical comparison of ILI distributions that incorporates each individual ILI, we performed a log-rank survival analysis on 500 ILIs per animal using data from 5WT and 5 *Fmr1*-KO mice. Thus, a total of 2500 ILIs from each group were analyzed. Fourth, the number of licks a mouse performed during each visit to the waterspout was counted. A visit was defined as a single lick or a train of licks preceded by a lick-free period of at least 60 s. Fifth, the number of lick bursts per visit to the waterspout was counted. A lick burst was defined as a single lick or a train of licks preceded by a lick-free period of at least 0.5 s. Next, the number of licks per burst and the distribution of intervisit interval durations were measured. Finally, as a measure for circadian changes in water consumption, we counted the number of licks performed during the active dark and the less-active light period and calculated the ratios. For this analysis, we used 48-h data from 5 WT and 5 *Fmr1*-KO mice recorded during the same session.

Rotarod Performance

On the day prior to data acquisition, mice were acclimated to a Rotamex-5 Rotarod (Columbus Instruments, Columbus, OH) rotating at 5 rpm for 5 min. No data were collected during acclimatization. On each day of testing, the assessment began at a rotation velocity of 1 rpm, with an acceleration rate of 1 rpm/5 s to a target of 50 rpm. Mice were given three trials at the same time each day for three consecutive days.

Elevated Beam Task

Mice were acclimated to crossing a 80-cm-long wooden square beam (12-mm width) elevated 50 cm above a padded base. A 60-W lamp located at the start platform served as an aversive stimulus, whereas the opposite end of the beam entered a darkened escape box. Mice were placed on the start platform and allowed to traverse the beam to the escape box at the opposite end. The time needed to cross the entire length of the beam was measured and the number of paw slips was counted. Each mouse repeated the test 3 times for both the 12-mm and 8-mm square beams.

Footprint Analysis

Mouse paws were dipped in nontoxic water-based paints (forepaws in green and hindpaws in red). Mice were then allowed to walk down an enclosed runway lined with white paper. Three trials were performed on 3 separate days within a 1-week period. Steps from the middle portion of each run were scanned to a digital image and measured for (i) hind-stride length, (ii) fore-base width (the distance between the right and left forelimb strides), (iii) hind-base width (the distance between the right and left hind limb strides), (iv) forepaw splay, and (v) hindpaw splay (Powell, Anch, Dyche, Bloom, & Richter, 1999) using ImageJ (National Institutes of Health, <http://rsbweb.nih.gov/ij>). A total of nine strides were analyzed for gait analysis, three consecutive strides from each trial.

Vertical Rope-Climbing Assay

Mice were acclimated to climb a vertical 40-cm-long, 10-mm-thick nylon rope prior to testing. The bottom of the rope was suspended 15 cm above a padded base and the top entered into a darkened escape box. A 60-W lamp was placed near the bottom of the rope to serve as an aversive stimulus. During acclimatization and testing, mice were placed near the bottom end of the rope with their heads up. Acclimatization consisted of four trials, with an intertrial interval of 5 min. This set of four trials was repeated 3 times. On the day of testing, three trials, with a 5-min intertrial interval, were completed for each mouse. The time needed to climb the rope and reach the escape box was measured.

Grip-Strength Analysis

To measure the grip strength, mice were held by the scruff of the neck with one hand and the base of the tail with the other hand. The mice were then brought close to a metal grid attached to a Columbus Instruments force meter and were allowed to grasp the grid as they were moved along the surface of the grid. Maximal strength (in grams) with which the mice pulled the grid against experimenter resistance was measured in triplicate trials, with a minimal intertrial interval of 5 min.

Open-Field Anxiety Test

Mice were placed in a circular arena (2.5-m diameter with 68-cm surrounding walls) and allowed to explore freely while their movement path was tracked by an automatic video tracking system (Biobserve GmbH, Bonn, Germany). After each 30-min session, animals were removed, and the arena was thoroughly cleaned with 30% ethanol and allowed to dry for 10 to 15 min before the next trial began. The experimenter was not present for the

duration of the experiment. Measurements of luminance showed a light intensity of 57 lux in the center of the arena and 35 to 37 lux close to the wall. In an offline analysis of the exploratory path, we quantified the amount of time the mice spent in the open field relative to the time the mouse stayed close (i.e., within approximately 10 cm) to the wall. The level of anxiety in mice is positively correlated with the avoidance of open spaces, that is, to the amount of time spent in the open field versus close to the wall (Lister, 1990).

Statistical Analysis

Statistical analyses to compare quantitative behavioral measures of WT and *Fmr1*-KO mice were performed using either SigmaStat (version 3.5; Systat, San Jose, CA) or SAS (SAS Institute, Inc., Cary, NC) software. Statistical significance (α) was set at 0.05. Body weight was measured only once and this measurement was used to compare the two groups of mice with a one-factor analysis of variance (ANOVA).

Parameters derived from the long-term fluid-licking assay were analyzed using Student's *t* test if the data were normally distributed and had equal variance. Those conditions were satisfied for ILI duration, number of licks per visit, number of licks per burst, number of bursts per visit, and number of licks during the day-and-night cycle. The Mann-Whitney *U* test was used to compare median values of intervisit intervals. Log-rank survival analysis of ILI durations was performed on the first 500 licks from 5 WT and 5 *Fmr1*-KO mice. Thus, a total of 2500 ILIs per group were analyzed for differences in the probability of interval occurrence as a function of interval duration (i.e., "survival times").

In order to reduce the effects of extreme values, the raised-beam task vertical rope climbing assay, rotarod performance and grip strength measurements were performed in triplicate for each mouse, and the median was employed for subsequent statistical analyses. Then, ANOVA was used to compare *Fmr1*-KO and WT using these median values. In contrast, given that slips were rare events, total slips were counted and used for group comparisons with a nonparametric ANOVA. For the footprint analyses, a total of nine steps were measured, and the mean was calculated and used for comparisons of stride length, fore-base width, hind-base width, forepaw splay, and hindpaw splay with ANOVA. Given that the 12 measures of balance and appendicular motor functions analyzed here are likely correlated, a Bonferroni correction was made to maintain familywise error rate for this component of our study. No post hoc corrections were made for the session or Session x Genotype effects on rotarod performance.

Results

Body Weight

In humans, FXS is associated with accelerated prepubescent growth (Loesch, Huggins, & Hoang, 1995). Dölen et al. (2007) reported a corresponding phenotype in *Fmr1*-KO mice that showed a higher than normal weight gain starting at P26. We measured the body weight of each mouse before behavioral testing but found no differences between *Fmr1*-KO and WT mice. The mean weights were close to identical (WT $M = 28.67\text{g}$, $SE = 0.52$; *Fmr1*-KO: $M = 28.45\text{g}$, $SE = 0.47$).

Rhythmic Licking and Water Consumption Behavior

We found that *Fmr1*-KO mice had a significantly slower lick rhythm, that is, longer ILI durations, than their WT littermates (WT mean ILI duration = 102.76 ms, $SE = 1.01$; *Fmr1*-KO mean ILI duration = 107.98 ms, $SE = 0.82$; Student's t test: $t(26) = 3.99$, $p < .001$; Figure 1A). There was a significant difference in the variability (i.e., coefficient of variation) of the lick rhythms between WT and *Fmr1*-KO mice (WT mean CV = 0.20, $SE = 0.01$; *Fmr1*-KO mean CV = 0.24, $SE = 0.01$; Student's t test: $t(26) = -3.20$, $p = .005$). Survival analysis (Figure 1C) revealed significantly different ILI survival probabilities between *Fmr1*-KO mice and their WT littermates ($p = 0.001$).

On a longer time scale, licking behavior was organized in visits to the waterspout, and visits were organized in bursts of licks, which could consist of anywhere between a few to more than 100 licks. There was no significant difference between *Fmr1*-KO and WT mice in the average number of licks they generated per visit to the waterspout (WT $M = 49.37$, $SE = 2.84$; *Fmr1*-KO $M = 45.14$, $SE = 3.03$; Student's t test, $t(26) = 1.02$, $p = .317$; Figure 1B). Mice would typically generate several lick bursts during visits to the waterspout. There was no significant difference between *Fmr1*-KO and WT mice in the average number of bursts per visit (WT $M = 5.75$, $SE = 0.56$; *Fmr1*-KO $M = 6.62$, $SE = 0.42$; Student's t test, $t(26) = -1.26$, $p = .22$) or in the average number of licks per burst (WT $M = 20.33$, $SE = 1.33$; *Fmr1*-KO $M = 18.03$, $SE = 1.74$; Student's t test, $t(26) = 1.05$, $p = .304$). Intervals between visits could be several hours long, particularly during the mouse's inactive daytime hours. Comparison of intervisit interval (IVI) distributions revealed no difference between median IVIs of *Fmr1*-KO and WT mice (WT median = 5.88; *Fmr1*-KO median = 5.82; $U = 2929437.0$, $n(\text{big}) = 2404$, $n(\text{small}) = 2483$, $p = .264$).

Both WT and *Fmr1*-KO mice showed significantly higher numbers of licks during the dark period, that is, when they are highly active, than during the light or resting periods (WT mean-count light period = 588.3, $SE = 101.9$, mean-count dark period = 1181.2, $SE = 219.4$, Student's t test, $t(8) = -2.45$, $p = .04$; *Fmr1*-KO mean-count light period = 429.3, $SE = 112.1$, mean-count dark period = 1279.0, $SE = 208.13$, Student's t test: $t(8) = -3.59$, $p = .007$). There was no significant difference in light–dark lick-count ratios in WT and *Fmr1*-KO mice (WT $M = 0.54$, $SE = 0.12$; *Fmr1*-KO $M = 0.34$, $SE = 0.06$; $t(8) = 1.48$, $p = .176$), indicating that the circadian rhythm of fluid consumption behavior was not affected by the *Fmr1* mutation.

Motor Learning, Gait, and Balance

There was a significant effect of genotype on rotarod performance as *Fmr1*-KO mice stayed significantly longer on the rotarod than their WT littermates ($F_{1,21} = 7.97$, $p = .01$). There was also a modest overall effect of session, $F_{2,42} = 6.17$, $p = .0045$, $\epsilon_{H-F} = 0.95$, with improvements in rotarod performance from Session 1 through Session 3 (see Figure 2). However, there was no significant Genotype $\times$ Session interaction, $F_{2,42} = 0.15$, $p = .15$, $\epsilon_{H-F} = 0.95$.

Ataxic gait is a core clinical symptom of cerebellar deficits in humans, characterized by a wide stance and gait, and a reduced stride length. Gait analysis in *Fmr1*-KO and WT mice

showed that *Fmr1*-KO mice had essentially normal gait by all measures. The two experimental groups had close to identical measures on all parameters derived from footprint analysis (fore-base width, $F_{1,21} = 0.00019$, $p = .99$; hind-base width, $F_{1,21} = 0.026$, $p = .87$; stride length, $F_{1,21} = 0.74$, $p = .40$; forepaw splay, $F_{1,21} = 0.094$, $p = .76$; hindpaw splay, $F_{1,21} = 0.15$, $p = .70$; Figure 3).

The elevated-beam assay evaluates horizontal walking coordination and balancing ability. *Fmr1*-KO mice and their WT littermates performed equally well on the 12-mm-wide beam, $F_{1,21} = 1.53$, $p = .23$. In contrast, *Fmr1*-KO mice tended to traverse the more challenging 8-mm-wide beam more slowly than their WT littermates, $F_{1,21} = 7.94$, $p = .01$ (see Figure 4A). No significant differences were detected in the number of paw-slips during elevated-beam testing (8-mm-beam: $\chi^2 = 0.59$, $df = 1$, $p = .44$; 12-mm-beam: $\chi^2 = 0.36$, $df = 1$, $p = .55$).

Grip Strength and Rope Climbing

Because an abnormally weak grip could influence the outcome of motor tests such as rope climbing, beam crossing, and the rotarod, we evaluated the grip strength in *Fmr1*-KO and WT mice. The test showed almost identical grip strengths in *Fmr1*-KO and WT mice ($F_{1,21} = 0.01$, $p = .90$; Figure 4B), excluding deficits in motor power as a potential cause for functional differences. Performance in the vertical rope-climbing assay, quantified by the time it takes a mouse to reach the top of the rope, depends on hind and forelimb coordination as well as on muscle strength. *Fmr1*-KO and WT mice performed similarly in the rope-climbing test ($F_{1,21} = 1.65$, $p = .21$; Figure 4C).

Open-Field Anxiety Test

Fmr1-KO and wild-type mice spent close to identical proportions of their total exploratory time (30 min) in the center of the open field versus close to the wall (i.e., within 10 cm of the wall), indicating similar levels of anxiety (WT $M = 0.48$, $SE = 0.01$; *Fmr1*-KO $M = 0.47$, $SE = 0.02$).

Discussion

Oromotor Function

The main goal of our study was to examine whether a genetic mouse model of FXS expresses motor coordination and oromotor deficits, both of which are commonly observed in FXS patients. We show that mice lacking *Fmr1* expression have a significantly slower rhythmic fluid-licking movement and an increased variability in their lick rhythm compared to their WT littermates. *Fmr1*-KO mice otherwise have mostly normal motor performances, suggesting that loss of *Fmr1* expression in mice might selectively affect brain circuits involved in rhythmic oromotor behavior.

A previous study of rhythmic licking behavior in a *Ube3a*deficient mouse model of Angelman syndrome also revealed a significantly slower licking rhythm in *Ube3a*-deficient compared with WT mice (Heck et al., 2008). This finding is consistent with the suggestion by Zhao, Pak, Smrt, and Jin (2007) that these two disorders share both behavioral and molecular characteristics, possibly resulting in a common cerebellar neuropathology.

A Possible Neuronal Substrate for Oromotor Deficits

The neuronal mechanisms controlling human speech articulation or mouse oromotor behavior are poorly understood. However, several lines of evidence suggest that the cerebellum plays an important role in oromotor behavior in both species. In rats and mice, rhythmic fluid-licking behavior is widely dependent on the cerebellum (Bryant, Boughter, Gong, LeDoux, & Heck, 2010; Welsh, Lang, Sugihara, & Llinas, 1995). The cerebellum has been shown to be critically involved in rhythmic oromotor control for fluid licking in both mice (Bryant et al., 2010) and rats (Vajnerová, Zhuravin, & Brozek, 2000). Altered cerebellar anatomy has been shown to be associated with *Fmr1* deficiency. Using imaging techniques, Ellegood, Pacey, Hampson, Lerch, & Henkelman (2010) have found significantly reduced size of two deep cerebellar nuclei (medial and interpositus) in *Fmr1*-KO mice compared with WT. It has been shown that pharmacological inactivation of the deep cerebellar nuclei causes a significant reduction of licking frequencies in mice and rats (Bryant et al., 2010; Vajnerová et al., 2000). It is thus likely that the reduced volume of the medial and interposed nuclei of *Fmr1*-KO mice are responsible for the slower licking rhythm in *Fmr1*-KO mice.

Speech-articulation deficit is a common problem in FXS patients (Abbeduto, Brady, & Kover, 2007; Barnes, Roberts, Mirrett, Sideris, & Misenheimer, 2006; Newell, Sanborn, & Hagerman, 1983; Paul et al., 1987). The cerebellum is well known to play a crucial role in speech articulation (Spencer & Slocumb, 2007). Cerebellar neuropathologies are consistently found in FXS patients (Mostofsky et al., 1998; Zingerevich et al., 2009), which may partially contribute to speech articulation deficit in FXS patients. The oromotor deficit in *Fmr1*-KO mice described here and the speech articulation deficits in FXS patients might have common neuronal causes associated with cerebellar dysfunction.

Circadian Rhythm

Sleep abnormalities are frequently diagnosed in FXS patients, but the level of severity varies widely (Gould et al., 2000; Hagerman & Hagerman, 2002). Our data show no difference in the circadian rhythmicity of fluid-licking behavior between WT and *Fmr1*-KO mice. Both groups had significantly higher counts of licks during the dark compared with the light periods, and the ratios of light- and dark-period lick counts were not significantly different between WT and *Fmr1*-KO mice. This result suggests that there is no difference between *Fmr1*-KO and WT mice in the circadian rhythmicity of fluid licking. This is consistent with the results of Zhang et al. (2008), who studied circadian rhythmicity in FXS-related single and double mutants. The human FMR1 gene has two paralogs in mice, the *Fmr1* and *Fmr2* genes. Zhang and colleagues (2008) monitored running activity in *Fmr1*-KO, *Fmr2*-KO, *Fmr1/Fmr2* double KO, and *Fmr1*-KO/*Fmr2* heterozygous mice over a 48-hr period and reported a complete absence of circadian rhythmicity in locomotor activities in *Fmr1/Fmr2* double knockout and *Fmr1*-KO/*Fmr2* heterozygous mice. *Fmr1*-KO and *Fmr2*-KO mice had normal circadian locomotor activities when kept in a 12-hr light–dark cycle but displayed a shorter free-running period of running activity when kept in total darkness. Our *Fmr1*-KO mice were kept in a 12-hr light–dark cycle while we were monitoring their licking behavior. It is possible that they would show a shorter free-running period of licking activity if kept in

complete darkness. Our conclusion about a normal circadian rhythmicity in licking behavior is thus limited to the 12-hr light–dark cycle conditions.

Standard Motor and Anxiety Tests

Our evaluation of overall motor performance and motor learning failed to reveal prominent motor power, coordination, or motor learning deficits in *Fmr1*-KO mice. With the exception of the narrow-elevated-beam task, *Fmr1*-KO mice performed equally well as their WT littermates on all standardized motor tests (rotarod, rope climbing, gait analysis, grip strength). The fact that *Fmr1*-KO mice needed longer than WT mice to cross a narrow elevated beam than their WT counterpart could be due to deficits in balance and limb coordination. However, *Fmr1*-KO animals did not show more paw-slipping incidents than WT mice. Hence it is unlikely that major balance or limb coordination deficits were the primary causes for the increased time. Additionally, we did not find any deficit in gait or grip strength, both of which could have negatively affected the beam-crossing performance of *Fmr1*-KO mice. Another possibility is that *Fmr1*-KO mice might suffer from increased anxiety, which is common in FXS patients (Hagerman et al., 2002). We performed a standard open-field test of anxiety and found no indication of increased anxiety in *Fmr1*-KO mice. This does not exclude the possibility of increased fear of height in *Fmr1*-KO mice, which we did not evaluate.

Reduced levels of anxiety have been reported in *Fmr1*-KO mice bred on both the C57BL/6J strain background (Peier et al., 2000) and the FVB (FVB_NJ-*Fmr1*^{tm1Cgr}) strain background (Qin, Kang, & Smith, 2002). The FVB strain used by Qin and colleagues (2002), however, suffered from blindness due to retinal degeneration, which may account for the increased amount of time spent in the center of an open field. By contrast, Restivo et al. (2005) reported increased anxiety measures (also based on open field tests) in *Fmr1*-KO mice bred on the C57BL/6J strain background. Anxiety levels were brought back to wild-type levels if *Fmr1*-KO mice were raised in an enriched environment. This manipulation did not change the anxiety levels in wild-type mice. Even though our mice were bred on the FVB strain background (FVB.129P2-*Fmr1*^{tm1Cgr}/J), we can exclude blindness as a contributing factor because this strain is homozygous for the 129P2/OlaHsd wild-type *Pde6b* allele and does not suffer from blindness due to retinal degeneration. It seems clear from these multiple studies that the level of anxiety in *Fmr1*-KO mice depends on genetic background and environmental factors, suggesting that each behavioral study with FXS mouse models may need to include a test of anxiety.

Rotarod Performance

On the accelerating rotarod, *Fmr1*-KO mice performed better than their WT littermates, that is, they stayed longer on the rotating rod (see Figure 3). However, there was no overt effect of genotype on the rate of motor learning (i.e., Genotype x Session interaction). A possible explanation for our finding comes from a screening study for FMRP-regulated transcripts by Brown et al. (2001) which indicated that the Munc13 family of phorbol ester receptors is highly regulated by FMRP protein. In fact, the Munc13-3 family member is specifically expressed in the cerebellum and deficiency of Munc13-3 results in a rotarod phenotype that is surprisingly similar to the phenotype we observed for *Fmr1* mutants (Augustin et al.,

2001). Similar to *Fmr1*-KO mice, *Munc13-3* mutants also performed better than their WT littermates in the first few trials of rotarod testing, followed by a leveling off in performance that was attributed to slightly faster motor learning of the WT animals (Augustin et al., 2001). Together these results suggest that the rotarod phenotypes in the *Munc13-3* mutant mice and *Fmr1*-KO animals may have the same molecular cause as the regulation of *Munc13-3* transcript is potentially lost in *Fmr1*-KO mice.

Rotarod performance differences can also have nonneuronal causes. It is known that increased body weight correlates with decreased rotarod performance (Brooks & Dunnett, 2009). But our comparison of body weights did not reveal any difference between *Fmr1*-KO and WT mice. Thus, body weight can be excluded as a contributing factor to rotarod performance differences. Dölen et al. (2007) reported a higher than normal weight gain in *Fmr1*-KO mice, which would result in decreased latency on the rotarod for *Fmr1* deficient animals. This body weight discrepancy may be due to strain-specific effects for *Fmr1*-KO phenotypes and/or on differences in the age of the mice when weighed. Dölen et al. (2007) tested mutants bred on the C57BL/6 background and weights were measured at P26, whereas we tested mice on the FVB background when they were at least 2 months old. Even though not directly related to the rotarod performance, it is interesting that *Fmr1*-KO mice performed significantly better than WT controls in the initial trials of both Barnes maze and Morris water maze tests (Yan et al., 2004). Yan et al. (2004) suggested that the initial better performance of *Fmr1*-KO mice might be due to reduced anxiety in *Fmr1*-KO mice, as reported by Peier et al. (2000). However, in our mice, which were bred on the FVB background, we did not find any difference in anxiety levels between *Fmr1*-KO and WT mice.

The fact that we found no significant motor defects in *Fmr1*-KO mice, except for a deficit in rhythmic fluid licking, is surprising and raises questions about the limitation of the *Fmr1*-KO mouse as an animal model for FXS. Although we tested a wide range of motor behaviors, it is possible that the standard motor tests employed here missed some motor deficits or that those deficits were too subtle to be detected. The new fluid-licking assay is specifically sensitive to oromotor deficits, a phenotype not tested by any of the standard motor tests, and is therefore a valuable addition to motor testing.

When working with mouse models, it is important to consider that the severity of motor (or other) deficits may also significantly vary with genetic background. A recent study by Spencer and Slocumb (2007) demonstrates that autistic-like behavioral deficits linked to the loss of *Fmr1* expression in mice depended significantly on genetic background.

In summary, *Fmr1*-KO mice manifest only minor, or no, abnormalities in appendicular motor coordination and balance. They do, however, show severe deficiencies in rhythmic oromotor movements. Notably, deficits in rhythmic fluid-licking in mice have been linked to cerebellar pathologies, and our fluid-licking assay is a sensitive and comparatively specific test for cerebellar function in mice (Bryant et al., 2010). However, additional work will be required to determine if these rhythmic oromotor deficits in the *Fmr1*-KO mouse model are, in any way, linked to cerebellar pathology and associated articulation deficits in humans

with FXS. In addition, further studies will be needed to determine if genetic background plays a role in the severity of motor deficits in *Fmr1*-KO mice.

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Figure 1.

The rhythm of fluid-licking movements was significantly slower in *Fmr1*-KO mice, but long-term aspects of fluid consumption behavior were similar in *Fmr1*-KO and WT mice. (A) The average ILI duration was significantly increased and hence the rhythm was significantly slower in *Fmr1*-KO compared to WT littermates. (B) Variability in lick rhythm as determined by the coefficient of variation (CV) ILI is higher in *Fmr1*-KO mice compared with their WT littermates. Error bars represent standard error of the mean (*SE*). (C) Log-rank survival plot of mean ILI shows that dynamics of the drinking pattern of FXS mice is different compared with their WT littermates.

Figure 2. *Fmr1*-KO mice stayed longer on the accelerating rotarod than their WT littermates and showed no deficit in motor learning. Error bars represent standard error of the mean (*SE*).

Figure 3. Footprint analysis of gait patterns showed no difference between WT and *Fmr1*-KO mice. (A) Fore-base width, hind-base width, and stride length. (B) Forepaw and hindpaw splay angles. Error bars represent standard error of the mean (SE).

Figure 4.

Beam-crossing grip strength and rope-climbing tests reveal only minor motor impairment in *Fmr1*-KO mice. (A) WT and *Fmr1*-KO mice crossed a 12-mm-wide square beam in about the same amount of time, but *Fmr1*-KO mice needed more time to cross an 8-mm-wide square beam than their WT littermates. (B) *Fmr1*-KO and WT mice had very similar grip strengths. (C) Rope-climbing performance was also quite similar between WT and *Fmr1*-KO mice. Error bars represent standard error of the mean (SE).