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Abstract

Fragile X syndrome (FXS) is a common inherited disorder that significantly impacts family and patient day-to-day living across the entire lifespan. The childhood and adolescent behavioral consequences of FXS are well-appreciated. However, there are significantly fewer studies (except those examining psychiatric comorbidities) assessing behavioral phenotypes seen in adults with FXS. Mice engineered with a genetic lesion of *Fmr1* recapitulate important molecular and neuroanatomical characteristics of FXS, and provide a means to evaluate adult behavioral phenotypes associated with FXS. We give the first description of baseline behaviors including feeding, drinking, movement, and their circadian rhythms; all observed over 16 consecutive days following extensive environmental habituation in adult *Fmr1*-KO mutant mice. We find no genotypic changes in mouse food ingestion, feeding patterns, metabolism, or circadian patterns of movement, feeding, and drinking. After habituation, *Fmr1*-KO mice demonstrate significantly less daily movement during their active phase (the dark cycle). However, *Fmr1*-KO mice have more bouts of activity during the light cycle compared to wildtypes. In addition, *Fmr1*-KO mice demonstrate significantly less daily water ingestion during the circadian dark cycle, and this reduction in water intake is accompanied by a decrease in the amount of water ingested per lick. The observed water ingestion and circadian phenotypes noted in *Fmr1*-KO mice recapitulate known clinical aspects previously described in FXS. The finding of decreased movement in *Fmr1*-KO mice is novel, and suggests a dissociation between baseline and novelty-evoked activity for *Fmr1*-KO mice.

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CONFLICTS OF INTEREST

STUDY ROLES AND CONTRIBUTIONS

Keywords

Fragile X syndrome; *Fmr1*-KO mouse; Behavior; home cage; Behavior; water ingestion; Behavior; activity; Behavior; locomotion

INTRODUCTION

Fragile X Syndrome (FXS) is the most common inherited intellectual disability and a leading monogenic cause of autism, affecting 1 out of every 4,000 males and 1 out of every 7,000 females (Lozano *et al.*, 2014). FXS is caused by an expansion of a CGG repeat in the 5′-untranslated region of the fragile X mental retardation 1 (*FMR1*) gene which results in silencing of the gene (Pieretti *et al.*, 1991). The fragile X mental retardation protein (FMRP) is expressed throughout the body, but in the brain FMRP is enriched in neurons (Devys *et al.*, 1993). FMRP regulates mRNA translation and transport and many of its targets are synaptic proteins (Darnell *et al.*, 2011; Ascano *et al.*, 2012). Individuals with FXS have cognitive (Van der Molen *et al.*, 2010) as well as behavioral impairments that include hyperactivity, attention deficits, social anxiety, sensory hypersensitivity, autistic-like behaviors such as gaze avoidance, perseverative language and hand stereotypies, motor skill deficits as well as speech impairments (Lozano *et al.*, 2014; Zingerevich *et al.*, 2009). Much of these behavioral studies have focused on children and adolescents. Published reports describing phenotypic characteristics of adults with FXS are mostly lacking, but do demonstrate (particularly in males) deficits in functional status, impaired activities of daily living performance, and increased risk for psychiatric disorders (Hartley *et al.*, 2011; Sabaratnum *et al.*, 2003; Bailey *et al.*, 2012; Smith *et al.*, 2012).

The *Fmr1*-KO mouse recapitulates some of the physical, neurological, and behavioral deficits observed in FXS. These features include macroorchidism (Bakker *et al.*, 1994), hyperactivity (per open field test; Kazdoba *et al.*, 2014; also Mineur *et al.*, 2002), attention deficits (Moon *et al.*, 2006), altered anxiety-related behaviors (Kazdoba *et al.*, 2014; Saré *et al.*, 2016), impaired social communication (Mineur *et al.*, 2006), sensory filtering deficits (Frankland *et al.*, 2004), motor deficits (Padmashri *et al.*, 2013; Hodges *et al.*, in press), and subtle cognitive impairments (Bakker *et al.*, 1994). Further studies have replicated *Fmr1*-KO subtle cognitive impairments (in water maze reversal task; D'Hooge *et al.*, 1997; radial arm task Mineur *et al.*, 2002; in fear memory Zhao *et al.*, 2005, in five-choice serial reaction time test, Krueger *et al.*, 2011). These studies demonstrate that *Fmr1*-KO mice are valuable models of human FXS useful for elucidating disease mechanisms and potential therapeutic interventions.

As the current evidence suggests that males with FXS have significant impairments in behaviors required for day-to-day living, we propose that mouse home cage behaviors provide an appropriate means to study these functional impairments. Here, we report the first characterization of daily behavioral patterns for adult *Fmr1*-KO mice in an acclimated home cage environment. Specifically, we compared both daily overall and within day temporal patterns of activity, locomotion, feeding, and drinking in *Fmr1*-KO and wildtype (WT) littermate mice. These studies demonstrate that functional loss of FMRP evokes a

well-defined phenotype characterized by: (1) decreased dark cycle water ingestion accompanied by tongue movement dyscoordination, (2) decreased locomotor movement, (3) increased light cycle activity bouts, and (4) unchanged overall feeding and metabolic behaviors.

METHODS

Mice

Fmr1-KO mice (C57BL/6) generated by homologous recombination at *Fmr1* exon 5 to create a null allele, were a gift of D. Nelson (Baylor). These mice were backcrossed to C57BL/6 for over 15 generations at the time of receipt and several times more in our own lab. For these experiments heterozygous females were mated to *Fmr1*-KO males to produce litters with males whose genotype was either WT or *Fmr1*-KO. Genotypes were determined by PCR analysis of DNA extracted from tail samples using previously described primers (Bakker *et al.*, 1994). Animals were cared for in accordance to NIH guidelines for laboratory animal welfare. All experiments were conducted with approval of the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Animals were raised on a 12-hour-on/12-hour-off light/dark cycle (lights on at 0600 CST), and were given food and water *ad libitum* (except where noted otherwise). Mice not undergoing testing were group-housed in the mouse housing facility. For this experiment, we tested 32 mice in total. Wildtype mice (WT) are littermates of the *Fmr1*-KO mice. 16 male mice (8 WT, 8 *Fmr1*-KO) were tested in one cohort and 16 male mice (8 WT, 8 *Fmr1*-KO) were tested in a second cohort. Mice were counterbalanced such that cages containing a WT mouse for the first cohort had a *Fmr1*-KO mouse for the second cohort, and *vice versa*. Mice had *ad lib* access to powdered chow (PicoLab Mouse Irradiated 5058, OH) and autoclaved water (prepared in house). Facility lighting duty cycle was set as above. Facility lighting intensity averaged at 1270 lux (Li-210SA, LiCOR, NE); room temperature ranged between 22.8 and 24.4°C and relative humidity between 5–40% (Watchdog V5.11, Edstrom Inc., WI). Facility walls were lead-lined, and completely blocked noise from the surrounding hallways and rooms. Entrance was key controlled and limited to two investigators (TRC, SJB).

Home cage monitoring

At the start of testing, the mice were 11–12 weeks old. Baseline mouse day-to-day behavior was measured using a custom-designed home cage monitoring system (HCM) that measures behaviors with 1 ms temporal and 0.5 cm spatial resolution (similar to that described in Goulding *et al.*, 2008). With the HCM, we simultaneously measure patterns of feeding, drinking, and movement in 32 individually housed animals for extended durations. After the mouse is introduced to the home cage, it receives no further human handling until the end of the experiment, 21 days after initial placement. Since mouse handling is a known stressor well-demonstrated to alter many behaviors (Balcombe *et al.*, 2004), we thus capture feeding, drinking, and movement behaviors without imposing significant external stressors on the mice. Our experience further shows that behavioral data streams for C57BL/6 mice taken with this system are highly similar regardless of system location or investigator (Tecott, Goulding, personal communication).

To measure licking, we used a custom lickometer that determined changes in capacitance as the mouse tongue made and withdrew contact from a stainless steel sipper tube. As referenced from an aperture milled in the cage wall, the lick spout tip protruded 0.5 mm into the cage. Our validation tests showed that with no mice in the system, no lick events were observed for any system cage. We also noted excellent agreement in lick events determined by our lickometer compared to manual scoring of high speed video while a mouse was licking (Parkison *et al.*, 2011). The capacitive system easily identifies missed licks (since an electrical connection was not established). However, on occasion a "water bridge" would extend from the sipper tube end to the mouse mouth, leading to an artifactually long lick duration. Mice manipulating the lick spout with their paws is another potential cause of long lick durations. Pilot experiments demonstrated that approximately one lick every 1200 licking events was longer than our 200 ms duration criteria. Given this relative scarcity, we thus decided not to censor lick durations >200 ms long from the dataset. The lickometer was Schmitt-triggered to limit output voltages to TTL low (0.2 V) and high (5.2 V); this output was sampled at 1 kHz. For our studies, the interlick interval is the time between the offset (TTL voltage high to low) of the nth lick, and the onset (TTL voltage low to high) of the (n +1)th lick (*i.e.* off-on intervals). Prior studies have demonstrated that total mouse water intake can be accurately inferred from the total number of licks per day, and the corresponding duration of each mouse lick (Supplemental Data, Goulding *et al.*, 2008).

We measured mouse feeding using a photobeam placed in front of the powdered food supply. Mice were weighed before and after placement into the home cage monitoring system. We determined mouse position and movement within the cage by measuring movement-evoked torques at three points in the cage (front left and right corner, back center), and solving exact equations (with known mouse weight) for mouse position. All data was sampled at 1 kHz, and written to disk using a real-time computer (to prevent potential skipped data points). All mouse data underwent rigorous quality control to eliminate known spurious values (arising from blocked photobeams or sipper tubes, sudden changes in cage center of mass, *etc.*), followed by a data classification workflow to determine mouse active and inactive states, and mouse bouts of feeding, drinking, and movement (Goulding *et al.,* 2008). Finally, we note that it takes time to place each mouse in its home cage at the experiment start, and that all mice have to be in the system to begin data collection. We thus do not measure feeding, drinking, or movement from the moment the mouse is first placed in the cage. Our first day habituation metrics thus do not reflect feeding, drinking, or movement performed by the mice during this brief interval.

Movement classification

An unsupervised machine learning algorithm determines locomotor speed and turning angle criteria associated with forward locomotion and movement-in-place, respectively. We classify paths characterized as having faster speeds, smaller turn angles, and no pauses as forward locomotion. Conversely, we classify paths characterized as having slower speeds, larger turn angles, and/or pauses as movement-in-place. Total activity is then calculated from the sum of both locomotor and movement-in-place distances (Goulding *et al.*, 2008). In our studies, most locomotor distance arises from forward locomotion, with about 10 fold less distance from movement-in-place bouts.

Periodicity analysis

Circadian periodicities for feeding, drinking, and movement are determined per Lomb (1976), Scargle (1982). Circadian waveforms are calculated by summing sine waves corresponding to significant periodicities identified by the Lomb-Scargle algorithm, weighted by periodicity amplitude.

Metabolic studies

Cohorts per above. Mice were tested up to four days prior to home cage behavioral monitoring. Dual energy X-ray absorptiometry (DEXA) studies were performed by standard protocol (modified from whitelabs.org: DEXA (GE Lunar PIXImus) Scanning Protocol), using inhalational isoflurane anesthesia, a PIXImus scanner (GE Lunar, Inside/Outside Inc., WI), and Piximus 2.10 software. For DEXA studies, mice were removed from their group housing cage, weighed, briefly anesthetized, imaged, transferred to a holding cage to recover from anesthesia, and then returned to their original housing status.

Our indirect calorimetry system (open circuit system; Oxymax Equal Flow, Columbus Instruments, OH) consisted of an air pump, CO_2 sensor (range 0%–0.8%; resolution 0.002% CO_2; drift <20 ppm CO_2/hr), paramagnetic O_2 sensor (range 0–100%; resolution 0.002% of specified range; drift <0.06% of specified range per 24 hrs), air dryer, controller, 8 hermetically sealed indirect calorimetry chambers ($20.1 \times 10.2 \times 12.7$ cm^3, part 760M-D8, Columbus Instruments), chamber photocell bracket (1.27 cm between photocells), photocell controller (Opto M3, Columbus Instruments), and software (Oxymax for Windows 4.49). Indirect calorimetry measures were taken per standard protocol (Tso 2013); mice were fasted between 9:00 AM and 1:00 PM on the testing day, the system turned on at 9:00 AM, and given 3 hrs to equilibrate before calibration. Calibration gases were 100% N_2 and a mixture of 0.5% CO_2/20% O_2/79.5% N_2 (span). During system operation and data collection, we used room air as the input gas for each calorimetry chamber. Each station serially cycled through 5 min of data acquisition for the duration of data collection (1:00 PM to 5:00 PM). To determine basal metabolic rate, we averaged values obtained from the three epochs (15 min total) where each mouse demonstrated the least activity (as measured by photobeam breaks). Conversely, to determine activity-associated metabolic rate, we averaged values obtained from the three epochs where each mouse had the greatest activity. We performed ANCOVA analysis to determine significant differences in the metabolic parameters maximum oxygen uptake ($\dot{V}O_2$), global oxygen delivery (DO_2), oxygen output (O_2out), maximum CO_2 production, ($\dot{V}CO_2$), global CO_2 removal (DCO_2), CO_2 output (CO_2out), and heat generated as a function of body adiposity (Tschöp *et al.*, 2012). Bonferonni-corrected two-tailed t-tests were used to assess for differences in remaining DEXA parameters: bone mass density (BMD), bone mineral content (BMC), bone area (BArea), tissue area (TArea), ratio of soft tissue attenuation (R_{ST}), total tissue mass (TTM), and weight.

Statistical analysis

Given the large number of behaviors measured by our home cage system, we first controlled familywise error rates across all measures except longitudinal time series measures (*e.g.* multiple measures over circadian day). Unless stated otherwise, we used a false discovery

rate statistic (FDR, calculated per MAFDR, MATLAB) set at $p<0.05$ (Benjamini & Hochberg 1995; Storey 2002). Behaviors identified as significant in this analysis were subjected to further analysis to determine overall and circadian differences between WT and *Fmr1*-KO groups. Dark cycle/light cycle comparisons were performed using two-way analysis of variance (ANOVA), including terms for the effects of genotype and light cycle, and a genotype × light cycle interaction (implemented with anova2, MATLAB). This metric is identical to a repeated measure ANOVA with lighting cycle as the repeated measure and genotype as the independent measure. Circadian comparisons were performed using Student's t-test followed by Bonferonni correction for the number of longitudinal time measurements (usually 12). For event duration and inter-event interval studies, we developed Gaussian mixture models using custom MATLAB code. Briefly, values for mixture pi, mu, and sigma were initially guessed using kmeans, and then refined by estimation maximization (EM). We began by fitting 2 component mixtures, and added additional mixture components until we achieved a target likelihood ratio (1×10^{-6}).

RESULTS

The overall home cage behavioral phenotype of Fmr1-KO mice is characterized by less drinking and movement

Eleven home cage behaviors differed between WT and *Fmr1*-KO mice. We depict these differences in a volcano plot (Figure 1), a specialized scatter plot that visualizes both statistical significance and fold change over large replicate datasets. In this figure, each individual point represents one behavior measured between WT and *Fmr1*-KO mice; points above the dotted line demonstrate statistically significant differences ($p<0.05$, paired two-sided t-test). Points left of the left dashed line show two-fold reduction in behavior (WT compared to *Fmr1*-KO); points right of the right dashed line show two-fold increase in behavior (WT compared to *Fmr1*-KO). Thus, points above the dotted line and left of the left dashed line or right of the right dashed line, show behaviors where *Fmr1*-KO mice show at least a two-fold change in a specific behavior compared to WT mice that is statistically significant at $p<0.05$ or better. Results for all behavioral outcomes are provided in Supplemental Table 1. Of these 11 behaviors found significant after FDR familywise error rate correction, 6 represented phenotypic changes in movement (3 of which are independent aspects of movement), and 5 represented changes in water ingestion. We examine these phenotypes in greater detail below.

Fmr1-KO mice have decreased movement behaviors

On the first day of habituation (first 24 hours mice placed into home cage environment), there were no genotypic differences in locomotion (WT 757.6 ± 77 m; *Fmr1*-KO 768.5 ± 61 m, NS; dark cycle WT 547.3 ± 68 m, *Fmr1*-KO 549.1 ± 44 m; light cycle WT 210.4 ± 18 m, *Fmr1*-KO 219.4 ± 29.5 m; all NS). Over the 16 days following 5 full days of habituation, *Fmr1*-KO mice showed ~17% less dark cycle movement on a day-by-day basis compared to wildtypes (WT 542.4 ± 124 m; *Fmr1*-KO 450.2 ± 103 m; two-way ANOVA; genotype $F_{1,30,63}=3.39$, p NS; lighting cycle $F_{1,63}=595.9$, $p \ll 0.001$; genotype × lighting cycle interaction $F_{1,30,63} = 7.71$, $p<0.009$; Bonferroni corrected for 3 comparisons; Figure 2A). Decreased movement distance in *Fmr1*-KO mice results from significant decreases in

forward locomotion and movement-in-place bout onsets (particularly in the last 4 hours of the dark cycle), as well as a strong trend toward decreased forward locomotion speeds (Figures 2B–2G depict this finding for forward locomotion; analogous data for movement-in-place bouts not presented).

Frm1-KO mice locomotor paths also demonstrated evidence of mild ataxia compared to WT (Figure 2H). Using a bounding box area metric to measure locomotor straightness, we find that *Fmr1*-KO mice had larger bounding box areas (corresponding to less straight locomotor pathways followed from stop to stop; $p<0.001$).

Fmr1-KO mice ingest less water secondary to altered licking dynamics

On a day-to-day basis, *Fmr1*-KO mice drank approximately 14% less water compared to wildtypes (WT: 4.41 ± 0.69 g; *Fmr1*-KO 3.79 ± 0.65 g, $p<0.015$); this difference occurred during the dark cycle, when mice were most active (two-way ANOVA; genotype $F_{1,30,63} = 6.738$, $p<0.015$; lighting cycle $F_{1,63} = 529.5$, $p\ll0.001$; genotype × lighting cycle interaction $F_{1,30,63} = 2.325$, p NS; Bonferroni corrected for three comparisons; Figure 3A). No significant circadian differences were noted in drinking bout probability, intensity (mg water ingested per second), onsets, duration, or per bout water consumption (data not shown). This finding suggests an etiology in drinking performance, rather than drinking patterns. We thus examined mouse licking coefficients, defined as the water intake (in mg) per single lick event. We calculated group licking coefficients by averaging daily lick coefficients across all WT and *Fmr1*-KO mice (Figure 3B). The overall lick coefficient of *Fmr1*-KO mice was ~9% lower (1.02) compared to wildtype (1.12), suggesting that *Fmr1*-KO mice ingested less water per lick compared to WT ($p<0.003$; one-way ANOVA). *Frm1*-KO mice also have many more mouse-days with very low lick coefficients (<0.5) compared to WT. Visual inspection of daily behavioral time series show that *Fmr1*-KO mice (Figure 3C) have greater variability in both lick duration and interlick interval compared to wildtypes (Figure 3D). Thus, decreased water intake in *Fmr1*-KO mice is attributable to dysregulation of primary licking behavior, manifested as less water intake per lick and greater lick duration and interlick interval variability, ultimately leading to less water consumption on a day-to-day basis.

Fmr1-KO mice have more light cycle periods of activity

Given known changes in sleep associated with FXS, we analyzed data related to active and inactive states, correcting for the number of tests within this overall behavioral domain. Of note, *Fmr1*-KO mice had more active state onsets during the light cycle compared to WT mice (5.7 ± 1.4 WT; 7.7 ± 3.9 *Fmr1*-KO, $p<0.05$). Other activity parameters associated with the light cycle, including state duration, total movement, chow and water consumption did not differ between WT and *Fmr1*-KO mice.

WT and Fmr1-KO mice have no differences in food ingestion or metabolism

No genotypic differences were noted in total daily food consumption, dark cycle/light cycle food consumption, feeding time budgets, feeding bout distributions within active states, and feeding bout probability/intensity/onset-rate/duration/per-bout-consumption. DEXA studies revealed no genotypic differences in mouse percent adiposity, BMD, BMC, BArea, TArea,

R_{SA}, TTM, or weight. Similarly, no genotypic differences were found in indirect calorimetry measures of $\dot{V}O_2$, DO_2, O_2out, $\dot{V}CO_2$, DCO_2, CO_2out, or generated heat under either basal metabolic or activity-associated conditions. Thus, we identify no significant change in overall mouse feeding or energy expenditure secondary to loss of *FMRP* function.

WT and Fmr1-KO mice have no circadian differences in movement, feeding, or drinking behavior

The original manuscript describing behavioral phenotypes of *Fmr1*-KO mice noted no significant differences in circadian behavior as assessed by running wheel activity under a 12-hr-on, 12-hr-off lighting cycle (Bakker *et al.*, 1994). We replicate and extend this phenotype by demonstrating no major differences between WT and *Fmr1*-KO mice in circadian rhythms of movement, feeding, and drinking. There is no phase shift in movement, feeding, or drinking waveforms between WT and *Fmr1*-KO mice (Figure 4A–C). We note small, but statistically significant, differences in the normalized power of the ultridian rhythms for movement, feeding, and drinking by Lomb-Scargle periodograms (Figure 4D–F). Ultridian rhythms (periodicities shorter than 24 hours) are required to shape circadian onsets and offsets (Westermark *et al.*, 2013). The small differences in normalized power of ultridian rhythms between WT and *Fmr1*-KO mice account for the small changes in movement, feeding, and drinking waveforms depicted in Figure 4A–C. Since we did not study mice under epochs of constant darkness, we did not detect any evidence of changes in free running period as noted by Zhang *et al.* (2008).

DISCUSSION

We describe the first long-term baseline observations of feeding, drinking, movement, and circadian rhythm in *Fmr1*-KO mice. These observations were taken over at least 16 consecutive days using an automated home cage monitoring system that eliminated mouse handling over the experimental duration. We examined feeding, drinking, and movement events at high spatial and temporal resolution.

Dissociation of overall movement in novel vs. home cage environmental settings in male Fmr1-KO mice

We note that *Fmr1*-KO mice show 17% decrease in overall movement throughout the 16 day observation period compared to WT mice; decreased distance covered by forward locomotion and movement-in-place bouts equally contributed to this overall deficit. This locomotor deficit was fully apparent during the dark cycle, when mice are most active. Dissecting this phenotype suggested that decreased bout onsets, particularly during the last four hours of the dark cycle, was the most significant driver of this behavioral change.

Our results were somewhat unanticipated since a majority of prior studies in the related but different open field assay demonstrated increased locomotor activity in *Fmr1*-KO mice (Yan *et al.*, 2004; Peier *et al.*, 2000; Spencer *et al* 2005; Qin *et al.*, 2005; Spencer *et al.*, 2011; Pietropaolo *et al.*, 2011; Uutela *et al.*, 2012; Olmos-Serrano *et al.*, 2011; Thomas *et al.*, 2011; Pacey *et al.*, 2011; Liu *et al.*, 2011). Findings of unchanged (Zhao *et al.*, 2005; Spencer *et al.*, 2006; Pietropaolo *et al.*, 2011, Goebel-Goody *et al.*, 2012; Baker *et al.*, 2010;

Spencer *et al.*, 2011; Wrenn *et al.*, 2015), and even decreased (Fish *et al.*, 2013) open field locomotor activity in *Fmr1*-KO mice have also been reported. Open field performance of *Fmr1*-KO mice also varied with mouse background strain (Pietropaolo *et al.*, 2011; Spencer *et al.*, 2011). With the exception of Pietropaolo and colleagues (2011), all of these studies examined locomotor function during the light cycle, in a novel arena, for 120 minutes or less (a vast majority for 30 minutes or less) of observation time. Given large differences in the total observation time between home cage and open field observations, mouse habituation to a novel arena may thus be a significant factor underlying the above observations. While we could not determine mouse habituation immediately after placement in the home cage monitoring system, we did note that during the first 24 hours of testing both WT and *Fmr1*-KO mice demonstrated increased home cage activity (compared to activity after acclimation), with no genotypic differences noted, findings concordant with above (Pietropaulo *et al.*, 2011). The one study that did measure locomotor activity for 24 hrs did not observe increased locomotion in *Fmr1*-KO mice bred to a C57BL/6 background (Pietropaolo *et al.*, 2011); these mice were of the same genotype used in our study.

Mouse husbandry conditions may also influence our observed phenotype. We individually housed mice for the duration of their metabolic and home-cage behavioral testing, a period of approximately 25–28 days. It is well-established that wild male mice prefer solitary social situations (Silver, 1995). In laboratory conditions, singly-housed mice may show a tendency toward increased activity (Voikar *et al.*, 2005; Guo *et al.*, 2004), with other studies demonstrating no significant differences in locomotor behaviors (Arndt *et al.*, 2009; Palanza *et al.*, 2001). While single housing has the potential to differentially affect *Fmr1*-KO mice compared to wildtypes, the above studies suggest that single housing is not the sole factor responsible for our observed phenotype. We therefore believe that prolonged home cage monitoring in acclimated and undisturbed mice over 16 observation days revealed behaviors that were not previously appreciated. These data further suggest that constitutive loss of *Fmr1* function dissociates baseline movement behaviors from novelty/exploratory-driven movement behaviors.

Nevertheless, the reduced locomotor activity observed in our study is difficult to reconcile with the hyperactivity generally associated with FXS patients (Hatton *et al.*, 2002; Sullivan *et al.*, 2006; Kazdoba *et al.*, 2014). Interestingly, hyperactivity in males with Fragile X has been shown to change over development with decreased activity in very young children followed by increase in preschoolers followed by decrease in adolescent boys (Tranfaglia 2011; Gabis *et al.*, 2011; Hustyi *et al.*, 2014). Although information regarding hyperactivity in adults with FXS is lacking, our data suggest that hypoactivity might be observed in adults with FXS.

Functional impairments in Fmr1-KO mice may translate to human FXS clinical phenotypes

We expand upon the initial report of Roy *et al.* (2011) describing longer interlick intervals and increased interlick interval variability in *Fmr1*-KO mice. In these prior studies, lick waveforms were analyzed as spikes, and interspike intervals determined for all bouts of licking over 72 hours. Intervisit intervals were examined separately; as were lick counts across light and dark cycles. We examined all behaviors associated with water ingestion for

at least 16 days following a 5 day home cage acclimation. Loss of *Fmr1* function had a profound effect on lick duration. We demonstrate that much of the variability in mouse oromotor function arose from an increased frequency of both very short and long lick durations in *Fmr1*-KO mice. We confirm that the drinking deficit of *Fmr1*-KO mice is highly significant during the dark cycle, not significant during the light cycle, and that *Fmr1* loss does not have a strong influence on circadian rhythms of fluid consumption. Finally, our studies show that *Fmr1*-evoked oromotor dysfunction leads to significant functional differences in water consumption, with mutant mice ingesting 14% less water on a daily basis. Since mutant mice patterns of drinking bout initiation, duration, intensity, and intake are similar to those observed in WT mice, it would be important to test whether *Fmr1* loss increases the overall behavioral "cost" (*e.g.* effort) associated with water ingestion.

The functional impairments in mobility and drinking demonstrated in male *Fmr1*-KO mice may have a clinical parallel in human adults with FXS. Adult men with FXS demonstrate marked functional impairments, with most requiring activities of daily living (ADL) assistance, lacking a high school diploma, and having limited friends and social engagements (Hartley *et al.*, 2011). Greater than 20% of adult males with FXS have feeding difficulties unrelated to utensil use; greater than 10% of adult males with FXS have significant difficulties with both expressive and receptive aspects of speech. In adults, persons with FXS have a ten-fold increase in psychiatric comorbidity compared to the population at large (Sabaratnam *et al.*, 2003); 30% of females, and 50% of males take medications to treat anxiety complaints (Bailey *et al.*, 2012). Adults with both FXS and autism were more impaired in both communication and social reciprocity compared to controls with either FXS or autism (Smith *et al.*, 2012). Of note, up to 10% of adult males with FXS continued to report difficulty swallowing pills (Bailey *et al.*, 2012).

Oromotor dysfunction could also contribute to the language impairments exhibited by individuals with FXS (Newell *et al.*, 1983; Paul *et al.*, 1987; Barnes *et al.*, 2006; Abbeduto *et al.*, 2007; Gernsbacher *et al.*, 2008; Finestack *et al.*, 2009). In support of this hypothesis, *Fmr1*-KO mice demonstrate altered ultrasonic vocalizations (Gholizadeh *et al.*, 2014; Rotschafer *et al.*, 2012; Spencer *et al.*, 2011; Roy *et al.*, 2012). One brain region known to play a role in both human speech as well as mouse oromotor function is the cerebellum (Bryant *et al.*, 2010; Fujita *et al.,* 2008; Ackermann 2008; Spencer & Slocomb 2007). Indeed, cerebellar neuropathology and dysfunction have been described both in FXS (Mostofsky *et al.*, 1998; Greco *et al.*, 2011; Hazlett *et al.*, 2012) as well as in *Fmr1*-KO mice (Koekkoek *et al.*, 2005; Ellegood *et al.*, 2010).

In summary, we provide the first report of *Fmr1*-KO mouse baseline home cage behavioral phenotypes. These phenotypes provide critical context to interpret data arising from a variety of behavioral assays, including those evaluating motor, sensory, autonomic, cognitive, affective, and circadian phenotypes. Our results justify further studies of how *Fmr1* functional loss may differentially affect baseline versus novelty-induced movement and locomotion. We also demonstrate that home cage monitoring is an appropriate platform to evaluate treatments designed to ameliorate the locomotion and oromotor deficits accompanying *Fmr1* functional loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

Fmr1	fragile X mental retardation 1 homolog
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome
HCM	home cage monitoring system
TTL	transistor-transistor logic

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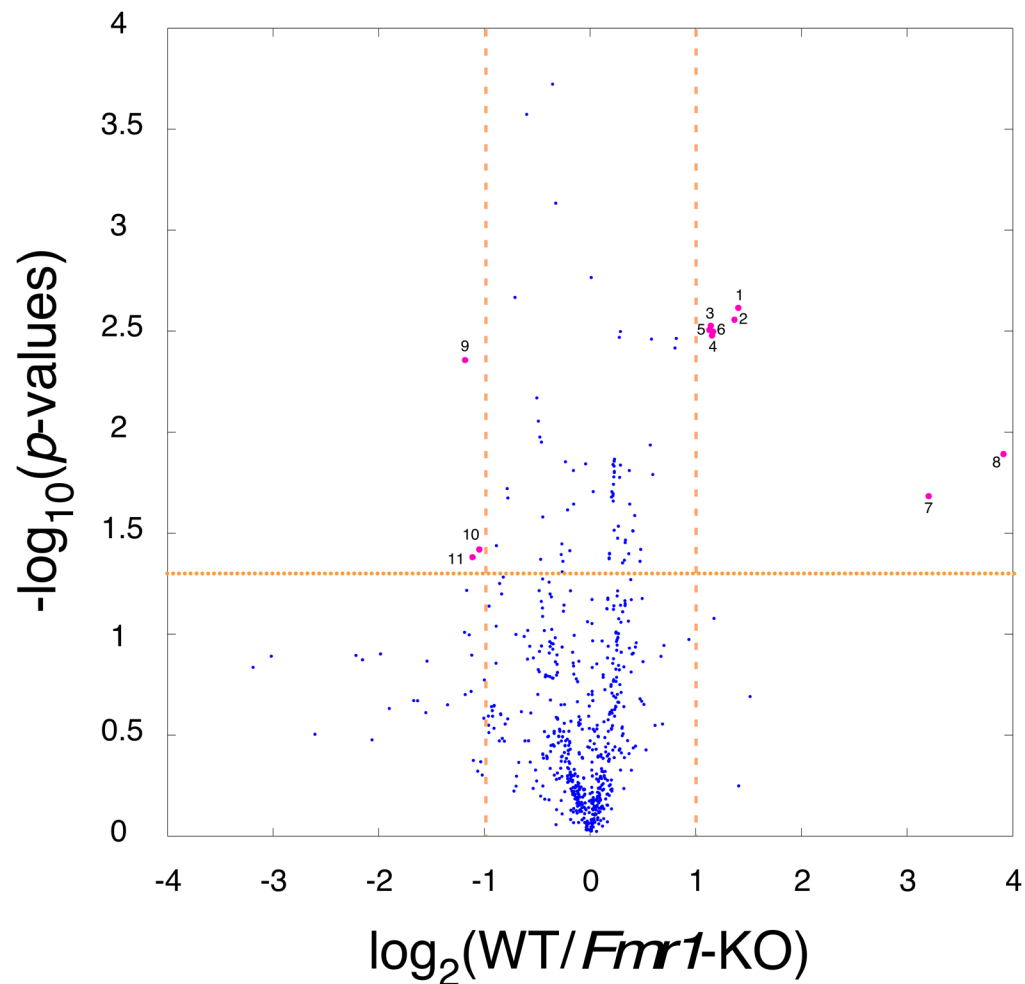
Figure 1. Volcano plot of differential behaviors between WT and Fmr1-KO mice controlling for familywise error rates across entire experiment

The *Fmr1*-KO mouse behavioral phenotype can broadly be characterized as having altered movement bout properties, and altered water consumption. Dashed vertical lines depict boundaries for two-fold decreases (left) and increases (right) in behavior (WT/*Fmr1*-KO); dotted horizontal line depicts behavioral significance $p<0.05$. Identified behaviors are: (1) movement-in-place bout rate dominance, (2) movement-in-place bout rate dominance per R^2, (3) stop dominance, (4) stop dominance per R^2, (5) forward locomotion bout rate dominance, (6) forward locomotion bout rate dominance per R^2, (7) percent of all active states during the light cycle containing no feeding, no drinking, and low movement, (8) percent of active states during light cycle containing no feeding, no drinking, and low movement, (9) number of active states during the light cycle that contained both small feeding and drinking bouts, (10) mean percentage of time stopped at the lick spout, (11) number of active states during the light cycle containing small feeding and large drinking bouts.
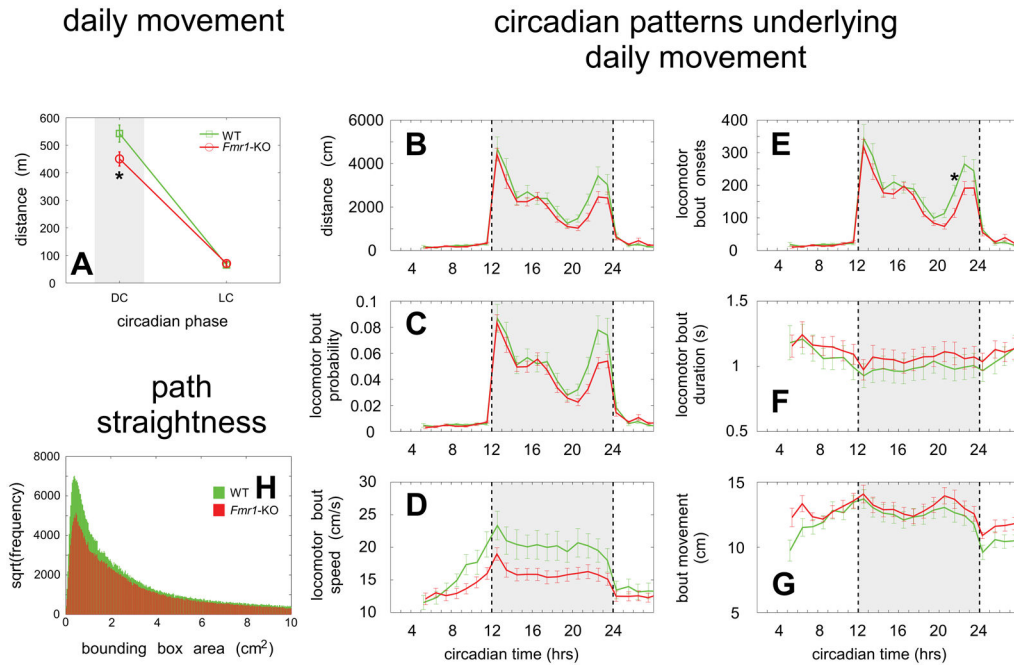
Figure 2. Fmr1-KO mice demonstrate less day-to-day movement secondary to decreased rate of movement bout onsets

A. Across entire experiment, *Fmr1*-KO mice have less movement during the dark cycle compared to WT controls. Green traces depict WT mice, red traces depict *Fmr1*-KO mice, * depicts $p<0.05$. **B–G**. Circadian patterns of forward locomotion bouts. **B**. Movement versus circadian time. **C**. Probability of a locomotor bout versus circadian time. **D**. Locomotor speed versus circadian time. **E**. Locomotor bout onset rate versus circadian time. **F**. Locomotor bout duration versus circadian time. **G**. Locomotor bout distance versus circadian time. For **B–G**, green traces depict WT mice, red traces depict *Fmr1*-KO mice, * depicts $p<0.05$ (Bonferroni corrected). Error bars ± 1 standard deviation. Shaded grey region depicts dark cycle activity; dashed lines correspond to lights off and lights on. **H**. Histogram of locomotor bounding box areas, transformed by square root. Lower bounding box values correspond to straighter locomotor paths. Median WT bounding box value 26.7; median Fmr1-KO bounding box value 27.9 (NS). Green bars depict values from WT mice; red bars depict values from *Fmr1*-KO mice.
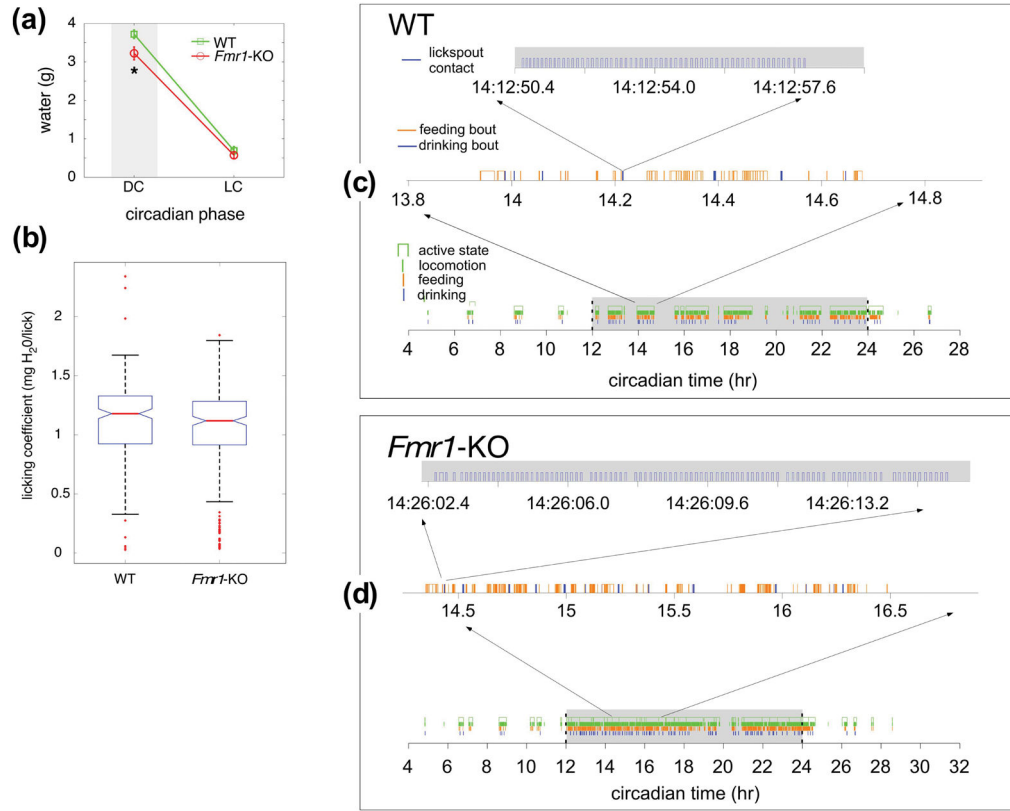
Figure 3. Fmr1-KO mice demonstrate deficits in water ingestion secondary to tongue dyscoordination

A. Across entire experiment, *Fmr1*-KO mice drink less water during the dark cycle compared to WT cohorts. Green traces depict WT mice, red traces depict *Fmr1*-KO mice, * depicts $p<0.05$. **B**. *Fmr1*-KO mice have decreased licking coefficients compared to WT. Boxplot of daily licking coefficients for WT (left) and *FMR1*-KO (right) mice. The central mark within each box depicts median, box edges are the 25th and 75th percentiles, the whiskers extend to the most extreme points not identified as outliers. Outliers depicted as red crosses above and below the whiskers. $p<0.003$ by one-way ANOVA. **C**. Representative licking bouts from WT mice. For each panel, the bottom set of traces depicts one day of active states (dark green rectangles), movement (light green hatches), feeding (orange hatches), and drinking (blue hatches). Dark cycle highlighted with grey background. The above set of traces zooms in on this representative active state. Blue and orange lines depict the onset and offset of drinking (blue) and feeding (orange) bouts. The top set of traces zooms in on a representative licking bout. **D**. Representative licking bouts from *Fmr1*-KO mice. Layout per panel **C**. Note that the WT mouse had less heterogeneity in both lick durations and interlick intervals compared to the *Fmr1*-KO mouse.
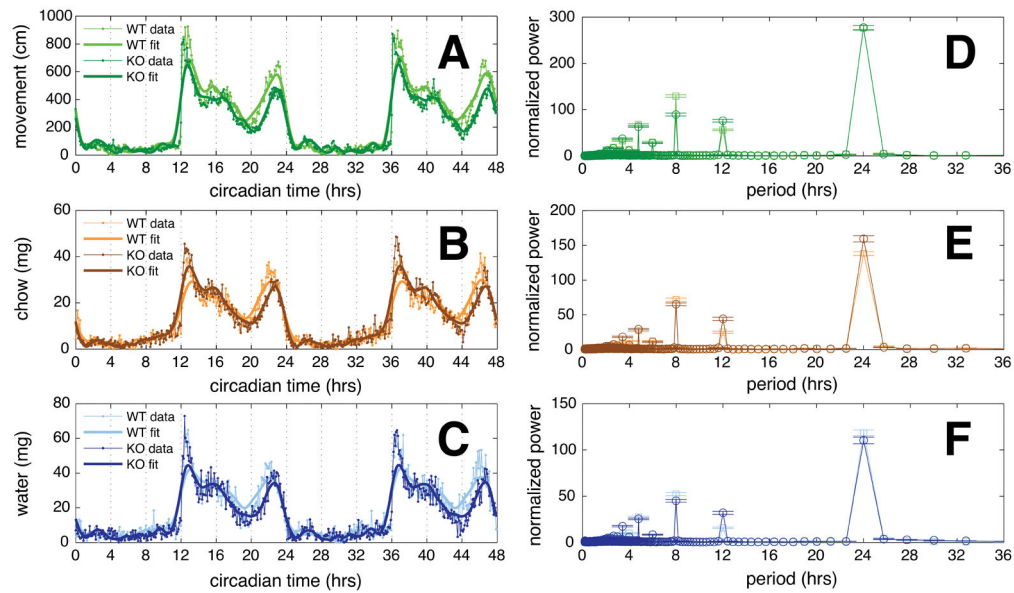
Figure 4. No significant differences in circadian rhythms for movement, feeding, and drinking between WT and Fmr1-KO mice

A. Observed and predicted movement for WT (light green) and *Fmr1*-KO (dark green) cohorts. Thin line with points depicts the mean values for each cohort; thick line depicts the predicted value (determined by summation of significant Lomb-Scargle periodicities). Note the increase in WT movement over the last 4 hours of the dark cycle. **B**. Observed and predicted feeding for WT (light orange) and *Fmr1*-KO (dark orange) cohorts. **C**. Observed and predicted drinking for WT (light blue) and *Fmr1*-KO (dark blue) cohorts. **D**. Lomb-Scargle periodogram for movement. WT periodogram in light green, *Fmr1*-KO periodogram in dark green. **E**. Lomb-Scargle periodogram for feeding. WT in light orange, *Fmr1*-KO in dark orange. **F**. Lomb-Scargle periodogram for drinking. WT in light blue, *Fmr1*-KO in dark blue. For **D**–**F**, error bars are ± 1 standard deviation. No significant periodicities of longer than 24 hours obtained for any behaviors.

SUPPLEMENTAL FILE

Table S1: *Fmr1*-KO and WT behavioral metrics. Column 1 lists the abbreviation for each behavior, column 2 lists the overall behavioral assay class, column 3 provides a brief description of the behavior, and column 4 is an index. Columns 5 and 6 list mean values for each behavior, control and *Fmr1*-KO, respectively. Columns 7–39 list results for each test by individual mouse. Control mice are listed in columns 7–22; *Fmr1*-KO mice are listed in columns 23–39. Columns 40, 42 and 44 provide unadjusted *P*-values calculated by Mann–Whitney, Student's *t*-test and fold change (B) methods. Columns 41 and 43 provide *P*-values adjusted by FDR for Mann–Whitney (column 41) and Student's *t*-test (column 43). Column 45 lists the behavioral fold change.

Excel spreadsheet, 267.7 KB

[File is attached to repository record home page for this document.]