Sexually dimorphic patterns in electroencephalography power spectrum and autism-related behaviors in a rat model of fragile X syndrome

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ABSTRACT
Fragile X Syndrome (FXS), a neurodevelopmental disorder with autistic features, is caused by the loss of the fragile X mental retardation protein. Sex-specific differences in the clinical profile have been observed in FXS patients, but few studies have directly compared males and females in rodent models of FXS. To address this, we performed electroencephalography (EEG) recordings and a battery of autism-related behavioral tasks on juvenile and young adult Fmr1 knockout (KO) rats.

EEG analysis demonstrated that compared to wild-type, male Fmr1 KO rats showed an increase in gamma frequency band power in the frontal cortex during the sleep-like immobile state, and both male and female KO rats failed to show an increase in delta frequency power in the sleep-like state, as observed in wild-type rats. Previous studies of EEG profiles in FXS subjects also reported abnormally increased gamma frequency band power, highlighting this parameter as a potential translatable biomarker. Both male and female Fmr1 KO rats displayed reduced exploratory behaviors in the center zone of the open field test, and increased distance traveled in an analysis of 24-h home cage activity, an effect that was more prominent during the nocturnal phase. Reduced wins against wild-type opponents in the tube test of social dominance was seen in both sexes. In contrast, increased repetitive behaviors in the wood chew test was observed in male but not female KO rats, while increased freezing in a fear conditioning test was observed only in the female KO rats. Our findings highlight sex differences between male and female Fmr1 KO rats, and indicate that the rat model of FXS could be a useful tool for the development of new therapeutics for treating this debilitating neurodevelopmental disorder.

1. Introduction

Fragile X Syndrome (FXS) is caused by mutations in the FMR1 gene. In most cases the genetic aberration is an expanded CGG repeat in the 5′ untranslated region of the gene causing the elimination or severe reduction of the encoded protein, Fragile X Mental Retardation Protein (FMRP). Fragile X mental retardation protein is an mRNA binding protein that regulates gene expression in the brain, testes, ovaries, and other organs. Within the central nervous system (CNS), FMRP is expressed at moderate to high levels in most brain regions. The clinical features of FXS encompass stereotyped behaviors, locomotor hyperactivity, sensory hypersensitivity, anxiety, impaired communication, and cognitive impairment. The syndrome is typically diagnosed in children around age 2–3 and predicts life-long disability (Erickson et al., 2018; Gallagher and Hallahan, 2012). Current pharmacotherapeutic treatments remain solely symptomatic with no disease modifying drugs available. Going forward, a key facet of the drug discovery process for FXS will be the full characterization and validation of additional animal models.

Each animal model of disease typically has its own set of pros and cons. For some neurological disorders, genetically modified rat models may be superior to mouse models in replicating human pathology, as in...
the case of Parkinson’s disease (Creed and Goldberg, 2018). Animal models of FXS include the drosophila, zebrafish, mouse, and rat knockout (KO) models, with the Fragile X Fmr1 KO mouse being the most widely used for both basic research and drug testing. Although Fmr1 KO mice have been very useful in the study of FXS, the Fmr1 KO rat represents a larger, genetically modified animal that could provide additional validity for drug testing, particularly on tests of cognitive abilities where rats may have a higher-level capacity compared to mice (Ellenbroek and Youn, 2016). Accordingly, genetically modified rat models of disease may be particularly applicable to neurodevelopmental disorders, such as in the study of several genetic forms of autism (Berg et al., 2018; Berg et al., 2020; Scott et al., 2018; Song et al., 2019).

A previously studied rat model for FXS has been generated on the Sprague-Dawley background strain. Differences were observed between wild-type and Fmr1 KO rats in memory/novel object tasks, and the Morris water maze (Tian et al., 2017; Till et al., 2015). While Sprague-Dawley rats are often used in behavioral studies, it has been shown that Long Evans rats require less training than Sprague-Dawley rats in operand tasks, making them potentially better suited for the complex cognitive tests required in testing mild impairments, such as those seen in rodent models of FXS (Turner and Burne, 2014).

The loss of FMRP can produce different effects in male and female rodent models (Nolan et al., 2017). While symptoms in female human patients are generally less severe than in males, due, in part, to maternal effects models (Nolan et al., 2017). While symptoms in female human patients are generally less severe than in males, due, in part, to maternal effects where rats may have a higher-level capacity compared to mice (Ellenbroek and Youn, 2016). Accordingly, genetically modified rat models of disease may be particularly applicable to neurodevelopmental disorders, such as in the study of several genetic forms of autism (Berg et al., 2018; Berg et al., 2020; Scott et al., 2018; Song et al., 2019).

2. Experimental procedures

2.1. Animals, breeding, and housing

Wild-type rats and Fmr1 KO rats on the Long Evans strain were generated as previously described (Asiminas et al., 2019). The animals were kept in a 12-h/12-h light/dark cycle with ad libitum access to water and food. Pups were weaned at postnatal day (PND) 21 and housed 2–4 to a cage.

2.2. Electroencephalography (EEG) recordings and data analyses

The methods for electrode construction and implantation were adapted from Jeffrey et al. (2013). Electrodes were made of bipolar twisted polyimide-insulated stainless steel wires with intracranial tips ~100 μm apart (0.10 mm outer diameter, Goodfellow Cambridge Limited, Huntingdon, England). The extracranial tips of the recording electrode were soldered individually to the two connecting pins ~4 mm apart. For the reference electrode, a twisted bipolar wire was soldered to a single connecting pin. The recording electrode was cut to 2 mm in length to target the frontal cortex while the reference electrode was cut to 1 mm in length for epidural positioning as described below.

For electrode implantation, the rat was anesthetized with 2–3% isoflurane and placed in a stereotaxic frame with a heating pad and the skull was exposed by skin incision. A thin plastic base was glued to the skull using a cyanoacrylate glue (Insta-cure+, USA) and two small holes (≤0.5 mm) were drilled through the plastic base and skull using a motorized mini drill at the following stereotaxic coordinates: left frontal lobe and/or right frontal lobe (4.7 ± 2.0) and right occipital lobe (–6.0, –2.0). The coordinates were relative to the bregma: anterior/posterior, medial/lateral (Supplemental Fig. 1). The recording electrodes were placed through the holes at the frontal cortex at a depth of ~2.5 mm according to the Rat Brain in Stereotaxic Coordinates by Paxinos and Watson (2006). Recordings from the left and right frontal probes were averaged for analysis. A reference electrode was placed in the occipital lobe epidurally. These coordinates were adapted from Lovelace et al. (2018). After the electrodes have been positioned, dental acrylic was overlaid onto the plastic base. Meloxicam (2 mg/kg) was dosed subcutaneously 20 min. before the surgery and once a day for two days after the surgery as analgesic.

2.2.1. EEG recording and data analyses

The EEG recordings took place 5–10 days after surgery between PNDs 38–72. EEG signals were recorded using a differential AC amplifier (Model 1700, A-M Systems, Carlsborg, WA, USA) and collected at an input frequency range of 0.1–1000 Hz with amplification gain at 1000×, and digitized at 5000 Hz (Digidata 1550B, Axon Instruments/Molecular Devices, Union City, CA, USA). Data acquisition and analysis were performed using pCLAMP software version 11 (Axon Instruments/Molecular Devices).

The rats were habituated to a plexiglass recording chamber for 15 min. before the extracranial connecting pins were connected to the amplifier via tethering wires through a slip ring located directly above the chamber. An additional 15 min was allowed for habituation to the tether before recording. Each rat was recorded for 3 h between 12 p.m. and 6 p.m. accompanied by video monitoring. To measure frequency power at “immobile awake” and “immobile sleep-like” states, artifact-free epochs (> 10 s) were selected based on the video recordings. An average of 30 ± 2 (mean, SEM) epochs was assessed per animal. The “immobile awake” state was defined as immobility accompanied by an upright head posture with eyes opened, while the “immobile sleep-like” state was defined as immobility accompanied by the rat laying down in a prone position with head down and eyes closed. EEG signals were analyzed only during immobility to minimize contamination by movement artifacts.

Fast Fourier Transforms were run on each epoch using 0.3 Hz bins with 50% window overlap and spectral power (µV²/Hz) was calculated from 0 to 100 Hz by pClampFit 11. Spectral power from each state in the 3-h recording were combined for analysis. To minimize inter-individual variability in signal strength, relative power was calculated for each frequency band (delta: 1–4 Hz; theta: 4–8 Hz; alpha: 8–12 Hz; beta: 12–30 Hz; and gamma: 30–100 Hz) by dividing the spectral power of a given band by the total spectral power across the entire frequency spectrum (0–100 Hz) (Sinclair et al., 2017; Thomas et al., 2017; Wang et al., 2017). To visualize the change in delta power, spectral data from the sleep-immobile state were normalized to the awake immobile data (average group value) as Normalized Power (Thomas et al., 2017). A 60 Hz notch filter was used to eliminate line noise and frequencies from 50 to 70 Hz were excluded in all analyses.

2.3. Behavioral analyses

Juvenile and young adult wild-type and Fmr1 KO rats were subjected to behavioral tasks measuring autism-related behaviors. All animals were age-matched, subjected to each test only once, and each test was carried out between 12 and 6 p.m., except for the wood chew test as described below. All equipment and surfaces were cleaned with 70% ethanol before and after each test.
2.3.1. Tube test for social dominance

The procedure for the tube test, conducted between PNDs 49–55, was modified from Arsenault et al. (2016), and Saxena et al. (2018). A transparent Plexiglas tube of 1 m in length was placed between two clean rat cages. Separate tubes of different internal diameter (7 cm and 6 cm) were used for the male and female rats, respectively. Rats were able to move freely but were unable to turn around or pass each other inside the tube. *Fmr1* KO rats were matched against an age and sex-matched wild-type opponent and each matched pair of opponents had never been housed together. Thirty minutes before the test, rats were transferred to the test room for habituation. Two rats were then tested by placing one rat at each holding cages and allowed to habituate for 15 min. before being placed into their respective ends of the tube and were released simultaneously. The rats generally met at the center of the tube and the match ended when one rat had both of its hind paws outside of the tube. The rat remaining in the tube was deemed the winner. Each matched pair was tested 3 times. The number of wins for each group was combined and Fisher’s Exact Test was used to determine

![EEG traces illustrating different behavioral states.](image)

(A) Representative EEG traces of different behavioral states from male and female wild-type and *Fmr1* KO rats. Power spectrum analyses and corresponding frequency band comparisons between males and females during the awake immobile state in wild-type (B) and *Fmr1* KO (C) rats. Significant differences (*) between the sexes were observed in both comparisons by 2-way ANOVA when the full spectrum (0–100 Hz) was compared (*p* < 0.05). No significant differences were observed in any individual frequency bands by post hoc analysis in both comparisons (B and C). Mean ± SEM were shown in the figures.
whether the percent of wins in each group was significantly different from the 50:50 win/loss outcome as expected by chance.

2.3.2. Elevated plus maze

The elevated plus maze test was conducted between PNDs 47 and 60. The maze was constructed as a raised cross of black Plexiglas, with a removable grey Plexiglas walking surface on the top of the cross. The walking surface was 50 cm off the ground, and walls of the closed arms were 40 cm high. The open and closed arms were 50 cm long and 10 cm wide, with a neutral center zone of 10 × 10 cm at the joining of the arms. Testing was performed at full light conditions; the rats were acclimatized to the testing room for 5 min. and then placed in the neutral center zone of the maze facing towards one of the open arms and allowed to move freely on the maze for 5 min. Movement and time spent in the closed arms, open arms, and the neutral center area were captured by a camera suspended over the elevated plus maze, and video was recorded and analyzed using Viewer 3 (Bioserve, Bonn, Germany). Time spent in open vs. closed arms was recorded, as was the number of visits to open vs. closed arms. Entering the neutral center zone and then into an arm increased the visit count for that arm by a value of 1.

2.3.3. Wood chew test

The wood chew test was performed as described in Hamilton et al. (2014) in male (PND 36–70, mean = 59.3 ± 3.3 SEM) and female wild-type and Fmr1 KO rats (PND 40–91, mean = 58.5 ± 4.0 SEM). The rats were single housed from 16:00 to 9:00 in a clean cage with standard bedding and a 1 in. x 1 in. x 1 in. wood block. The wood block was weighed before and after exposure. After the overnight exposure, the wood block was dried in a 37 °C incubator overnight to remove moisture from saliva or wet bedding before weighing. The difference in weight was calculated as mass chewed (g).

2.3.4. The nose poke test

The nose-poke apparatus consisted of a black opaque acrylic board inserted into an empty rat cage, with 9 equidistant holes of 3 cm in diameter and 7 cm in depth. The rats were placed individually into the apparatus and the number of nose pokes made during a 10 min. period was measured.

2.3.5. Startle response and prepulse inhibition tests

These tests were carried out on male (age PNDs 51–58) and female (age PND 51–59) wild type and Fmr1 KO rats (male WT: n = 11, male KO: n = 14, female WT: n = 9, female KO: n = 12). Testing was conducted using the SR-LAB Startle Response System (San Diego Instruments, San Diego, CA). The testing protocol consisted of startle stimulus only trials (120 dB; baseline startle response) and PPI trials (startle stimuli preceded by pre-pulses of different intensities: 74, 78 and 82 dB).

Fig. 2. Slow wave power spectrum patterns between awake and sleep states. Comparisons of slow wave power spectrum patterns between awake and sleep states in wild-type and Fmr1 KO male (A) and female (B) rats. The sleep-like state data (0-8 Hz) were normalized to the awake immobile state data. A comparison of behavioral states (awake/sleep) in the wild-type rats showed a significant increase (*) in the 1–2 Hz frequency range using raw spectral power data in both sexes (2-way ANOVA by frequency and post hoc Sidak multiple comparison, p < 0.05). This increase was not observed in the KO rats. The shaded area in each panel represents the S.E.M.
Fig. 3. Power spectrum analyses and corresponding frequency band comparisons.

Power spectrum analyses and corresponding frequency band comparisons between wild-type and Fmr1 KO male (panels A and B) and female rats (C and D) during awake and sleep states. Significant differences (p < 0.05) between genotypes (*) were observed in all four comparisons by two-way ANOVA when the full spectrum (0–100 Hz) was compared. Post-hoc analysis (Sidak’s multiple comparison test) revealed a significant increase in the gamma frequency range in the male Fmr1 KO rats during the sleep-like state and significant decrease in the theta frequency range in the female KO rats during the awake immobile state (p < 0.05). Means ± S.E.M. are shown.

2.3.6. Open field test

The open field test was carried out between postnatal days (PNDs) 50 and 64. The rats were tested in a 40 cm × 40 cm AccuScan locomotor activity testing apparatus (Omnitech Electronics, Columbus, Ohio), consisting of a clear plexiglass box with a white floor, and 16 location detection photobeams across both the x and y axes. Testing was performed under low light conditions. The rats were acclimated to the test room for 5 min. and then placed into the center of the open field chamber and the chamber was enclosed with a lid. Rats were allowed to freely explore the chamber for 20 min., without the researcher present, and locomotion was recorded with Fusion software (Omnitech Electronics, Columbus, Ohio). The data were split into 5 min. bins for analysis. The data were also split into zones, where Zone 1 was the outer perimeter of the field, and Zone 2 was the center area of the field, such that the width of Zone 1 against any side was equal to half the width of the center area (Zone 2).

2.3.7. Home cage activity analysis

Wild-type or Fmr1 KO rats were placed inside a plexiglass cage (44 × 31 × 55 cm) with food and water ad libitum. Each rat was recorded individually and was placed into the recording apparatus ~12 hours before recording began for habituation. Rat movements were recorded using a raspberry Pi 3 model B microcomputer (RS Components, Ltd.) equipped with a Smraza SMP 1080p OV5647 Video Webcam Night Vision camera from the top of the cage for 4 consecutive days starting from PND35-38. All videos were analyzed with DeepLabCut (version 2.1.6) to obtain the (x, y) coordinates of the rat's body location in pixels within the cage (Nath et al., 2019). Only coordinates with an associated likelihood score greater or equal to 0.95 were included in subsequent analysis. Euclidean distance is the length of the line segment connecting two coordinates and was calculated to quantify the rat’s movement in pixels between sequential (x, y) coordinates by repeatedly applying the Pythagorean theorem using the following formula (D’Souza et al., 2014):

\[ d(q,p) = \sqrt{\sum_{i=1}^{n} (q_i - p_i)^2} \]

where \( p \) and \( q \) were separate (x, y) coordinates generated by DeepLabCut in sequential frames. Afterwards the area under the curve (AUC) in the light phase (7 a.m. to 7 p.m.) and dark phase (7 p.m. to 7 a.m.) was determined by calculating the definite integral of each hour and summing the results. The following is the equation used to determine the AUC in the dark phase:

\[ \int_{t_{\text{pm}}}^{t_{\text{am}}} f(x) \, dx = \int_{t_{\text{pm}}}^{t_{\text{pm}}} (mx + b) \, dx + \int_{t_{\text{pm}}}^{t_{\text{pm}}} (mx + b) \, dx + \ldots + \int_{t_{\text{am}}}^{t_{\text{am}}} (mx + b) \, dx \]

where \( mx + b \) represents the linear equation between two sequential time points.

2.3.8. Fear conditioning

This test was conducted between PNDs 53 and 64. The test was conducted over two days in a soundproof chamber using the Ugo Basile rat fear conditioning system with rat cage (#46002, Ugo Basile, Verase, Italy). Data and video were recorded using the accompanying ANYmaze software (v6.06, Stoelting Co., IL, USA). Chambers and equipment were cleaned with 70% ethanol between tests/animals. On each day of testing the animals were brought in their home cages into a quiet staging room or hallway for 15 min., and then into the test room for 5 min. prior to testing. All testing was performed between 1 and 6 p.m. with white noise in the chamber, lit by white light. Fear conditioning consisted of a 2-day protocol, with Day 1 for conditioning/training, and Day 2 for testing of the conditioning.

2.3.8.1. Day 1

On the first day (training), the animals were placed into a test chamber with black walls, a metal wire floor, and vanilla extract was placed in a plastic weigh boat beneath the floor (Context A). The
animals were allowed to acclimate to the chamber for 3 min. and then exposed to a constant tone for 30 s. followed immediately by a 0.7 mA shock over 2 s.; this was followed 2 more training periods consisting of a 2 min. rest period, followed by exposure to the tone, and then the shock, for a total of 3 tone/shock pairs.

2.3.8.2. Day 2. Twenty-four hours after training, the animals were placed into the test chamber, under the same conditions as the training day (Context A). Freezing time was recorded over 3 min. The conditioned tone used during the training session on Day 1 was then played continuously for 3 min., and time frozen was recorded. Fear conditioning was assessed by measuring the total time spent frozen over 3 min. using the ANYmaze software freezing detection, with a minimum freezing episode threshold of 1 s. The data were analyzed via two-way ANOVA followed by Tukey’s post hoc test.

3. Results

3.1. Electroencephalography analyses

Previous studies have reported abnormally higher gamma spectral power in FXS patients and male Fmr1 KO mice (Lovelace et al., 2018; Wang et al., 2017). Here, we performed intracranial EEG recordings in the frontal cortex of the Fmr1 KO male and female rats; representative EEG traces are illustrated in Fig. 1A. In a comparison between the sexes, spectral power in the awake immobile state was found to be overall higher in the wild-type female rats than the wild-type male rats by 2-way ANOVA (Fig. 1B). No significant differences were observed in any specific frequency bands in a post-hoc analysis. In contrast, spectral power in the awake immobile state was found to be lower in the Fmr1 KO female rats than in the Fmr1 KO male rats (Fig. 1C).

An increase in slow delta wave activity is indicative of slow wave sleep (Corsi-Cabrera et al., 2001; D’Cruz et al., 2010). Spectral power in the sleep-like state (0–8 Hz) was normalized to awake immobile state; this analysis revealed an increase in delta waves in the wild-type rats...
A comparison of behavioral states showed a significant increase in the 1–2 Hz frequency range using raw spectral power data in both sexes (2-way ANOVA by frequency and post hoc Sidak multiple comparison, $p < 0.05$). In contrast, this difference was not observed in male or female Fmr1 KO rats, suggesting that the mechanism(s) that normally induces this increase delta wave activity was disrupted in the Fmr1 KOs (Fig. 2A and B).

Fig. 3 illustrates a comparison between the genotypes in EEG power across all frequency bands during the immobile awake and sleep-like states. In the male rats, a significant difference between genotypes was observed by 2-way ANOVA in both the awake and sleep-like states, but only the gamma frequency power in male KO rats during the sleep-like state was significantly higher by post hoc analysis (Fig. 3A and B). In the female rats, a significant difference between the genotypes was indicated by 2-way ANOVA in both the awake and sleep-like states, and the theta frequency power in KO rats during the awake immobile state was significantly lower by post hoc analysis (Fig. 3C and D). These results demonstrate sexually dimorphic EEG patterns in the Fmr1 KO rats.

3.2. Behavioral profile of the Fmr1 KO rat

3.2.1. Wood chew and tube test results

The wood chew test is a measure of stereotyped and repetitive behavior in rodents. Male Fmr1 KOs displayed elevated perseverative chewing behavior compared to wild-type rats (Fig. 4A), a result comparable to a previous report using a different strain of Fmr1 KO rats (Sprague-Dawley strain; Hamilton et al. (2014)). Interestingly, our results demonstrate that this behavior is not present in the female KOs.

The tube test is used to assess social dominance in rodents. In this test, a test animal is placed at one end of a tube while a control animal is allowed to enter simultaneously from the other end. Typically, one animal pushes the other out of the tube thereby establishing a winner and a loser, the latter being deemed the more submissive of the pair. Both male and female Fmr1 KOs were more submissive compared to sex and age-matched wild-type rats (Fig. 4B). These results are consistent with previous reports in Fmr1 KO mice (Gholizadeh et al., 2014; Spencer et al., 2005) and Fmr1 KO male rats (Saxena et al., 2018).

3.2.2. Open field test

The data from the open field test were calculated as the total distances travelled in 5 min. bins for a total of 20 min., within the entire field (Zones 1 + 2), the outer perimeter of the field (Zone 1), and the center area of the field (Zone 2, Fig. 5A). Male Fmr1 KOs travelled less distance in Zone 1, in Zone 2, and across the entire field, relative to wild-type controls (Fig. 4B). These results are consistent with previous reports in Fmr1 KO mice (Gholizadeh et al., 2014; Spencer et al., 2005) and Fmr1 KO male rats (Saxena et al., 2018).
center of the field (Zone 2) compared to wild-type female rats (Fig. 5D). These results indicate that male Fmr1 KO rats have reduced motor activity and/or exploratory behaviors in the open field test compared to wild-types, and the reduced distances traversed in the center zone by both sexes may suggest an elevated level of anxiety in the Fmr1 KOs.

3.2.3. Home cage activity

Home cage activity analysis was conducted to evaluate movement in the daily 12-h/12-h light/dark cycle. Both male and female Fmr1 KO rats showed a significant increase in movements ($p < 0.05$) compared to the wild-type rats in the 24-h day cycle (Fig. 6A and B) by 2-way ANOVA. Comparison between the area under the curve (AUC) in the light or dark phases found significant increase in activity ($p < 0.05$) during the dark phase but not the light phase in the female KO rats by 2-way ANOVA and post hoc Sidak's multiple comparison test (Fig. 6B). Activity in the nocturnal dark phase was also higher in the male Fmr1 KO rats but the results were not statistically significant ($p > 0.05$; Fig. 6A). We note that our results in the Fmr1 KO rat differ from activity...
comparison results reported in Fmr1 KO mice by Sare et al. (2017) who found increased activity during the light phase. However, in that study, the light/dark cycle was reversed which may have impacted the behavior.

3.2.4. Fear conditioning

The fear conditioning test probes fear and fear memory. The test animal is trained by repetition of a tone (the conditioned stimulus) followed by a floor shock in a particular identifiable context (Context A, Fig. 7A). Fear memory (quantified as percent time demonstrating an instinctive freezing behavior) is then probed by exposure to the conditioned context and tone. Neither male nor female Fmr1 KO rats displayed a significantly different rate in freezing relative to wild-type rats in response to the conditioned context (Fig. 7B). An increased rate of freezing in Context A (Fig. 7B) relative to the rate of freezing after exposure to a novel context (Context B, Fig. 7C) indicates that there is specific recognition of, and a fear response to the conditioned context (Context A). This lack of difference between groups suggests that there was no difference in hippocampal-dependent fear memory in Fmr1 KO rats relative to wild-type rats. When presented with the conditioned tone however, the female Fmr1 KO rats presented with an increased freezing rate relative to the wild-type females in the first minute of exposure (Fig. 7D). No difference was found between the male KO and wild-type rats in response to the conditioned tone. When adjusting for the inherent activity of each individual rat by subtracting the freezing rates in response to Context B alone from the freezing rates in response to the conditioned stimulus presented in Context B, Fmr1 KO females again show a phenotype of significantly increased freezing relative to wild-type females (Fig. 7E). These results suggest an increased level of fear in the KO females, an increased degree of fear memory in KO females, or both.

Several additional tests were performed but the results showed no consistent differences in wild-types vs. Fmr1 KO's. The elevated plus maze test is commonly taken as a measure of anxiety, with more time in the open arm and less in the closed arms interpreted as lower anxiety. However, in KO mice the opposite performance on this test (more time in the open arms and less in the closed) has been reported by several groups (Arsenault et al., 2016; Liu and Smith, 2009; Qin et al., 2011), prompting the suggestion that this test may be measuring impaired cognition in Fmr1 KO mice (Arsenault et al., 2016). However, in the Fmr1 KO rats we did not observe a consistent genotype effect on the elevated plus maze test (Supplemental Fig. 3). We also did not observe consistent patterns of abnormal behavior in the nose poke test probing exploratory behavior (Supplemental Fig. 4), and in the acoustic startle response and pre-pulse inhibition for assessing sensorimotor gating (Supplemental Fig. 5). The induction of sound-induced audiogenic seizures is a robust endophenotype in the Fmr1 KO mouse (Musumeci et al., 2000; Pacey et al., 2009). However, we were unable to induce seizures in Fmr1 KO rats under the same conditions that precipitated audiogenic seizures in Fmr1 KO mice (data not shown).

4. Discussion

We observed an overall higher EEG power in wild-type female compared to wild-type male rats in the awake immobile state. This result is consistent with previous studies in humans where females were found to have generally higher EEG power than males (Dijk et al., 1989; Jausovec and Jausovec, 2010; Wada et al., 1994). Notably, this difference was not observed in the female or male Fmr1 KO rats. We did not control for the phases of the menstrual cycle in the female rats during the behavioral tests and the EEG recordings. Although this might have been a confounding factor, previous studies have found higher EEG power in females vs. males where the female subjects were matched for the phases of the menstrual cycle (Jausovec and Jausovec, 2010), and in a study in gonadectomized rats where EEG power was also higher in the females (Del Rio-Portilla et al., 1997).

Compared to wild-type rats, we found that in the frontal cortex, male Fmr1 KO rats displayed an abnormal elevation in the higher frequencies (gamma power), while reduced power at lower frequencies was seen during the immobile sleep-like state in both male and female Fmr1 rats. This pattern is similar to that reported by Berzhanskaya et al. (2017) who recorded EEGs in the visual cortex of younger juvenile Fmr1 KO rats on the Sprague-Dawley background (male and female data combined). In that study, Fmr1 KO rats were found to have higher relative power in the higher frequency range (18–69 Hz) and lower power in the lower frequency range (3–13 Hz) during quiet wakefulness compared to wild-type rats. Sleep disturbances have been reported in FXS children and abnormalities in sleep architecture have also been found in Fmr1 KO mice (Boone et al., 2018; Kronk et al., 2010). A more detailed EEG study assessing sleep architecture in Fmr1 KO rats would be informative.

Greater gamma frequency band power in resting EEG has been observed in FXS patients and in male Fmr1 KO mice (Ethridge et al., 2017; Lovelace et al., 2018; Sinclair et al., 2017; Wang et al., 2017). Abnormal gamma power in FXS has been associated with hyperexcitability, attributed in part to excitatory/inhibitory imbalance (Cea-Del Rio and Huntsman, 2014; Goncalves et al., 2013). The increased gamma activity was observed in the male KO rats but not the female KO rats during the sleep-like state. We saw a different pattern in the female KO rats whereby they exhibited reduced theta power in the awake immobile state. Frontal theta power has been associated with cognitive activities that require attention and memory (Scheeringa et al., 2008). Repetitive and restrictive behaviors are a prominent feature of the clinical FXS phenotype. Consistent with the results from an earlier study of Sprague-Dawley Fmr1 KO rats (Hamilton et al., 2014), increased wood chewing was observed here in male Fmr1 KO rats on the Long-Evans strain. Repetitive behaviors are seen in highly arousing situations and have been proposed as a coping mechanism to alleviate anxiety (Oakes et al., 2016). We observed increased wood chewing in male KOs but not in female KOs. In humans, repetitive behaviors are observed in both males and females with FXS. In one study, however, the occurrence of sensory stereotypies was significantly higher in male FXS subjects than females, while other repetitive behaviors including hand mannerisms and stereotypic languages were similar between the sexes (Baker et al., 2019).

The tube test of social dominance was performed to assess social anxiety and withdrawal, another characteristic of FXS. Impaired social interaction was observed in the Fmr1 KO rats as indicated by the reduced number of wins against wild-type opponents in both male and female rats. This result is consistent with an earlier study using adult male Fmr1 KO rats of the same strain (Saxena et al., 2018). Reduced social dominance has also been found in Fmr1 KO mice, indicating that this is a consistent and robust behavioral feature of the Fmr1 KO rodents (Gholizadeh et al., 2014; Spencer et al., 2005).

Motor hyperactivity and anxiety are observed in most persons with FXS (Biag et al., 2019; Hustyi et al., 2014). Our results showed that both male and female Fmr1 KO rats displayed reduced exploratory behaviors in the center zone of the open field test suggestive of heightened anxiety. Previous work in male Fmr1 KO rats found no difference in locomotor activity relative to wild-type rats (Hamilton et al., 2014; Tian et al., 2017). This discrepancy may be due to the use of a different background strain (Sprague Dawley) compared to the Long Evans rats studied here. The results of the fear conditioning test were also suggestive of increased fear and anxiety, an effect that was more prominent in the Fmr1 KO females compared to KO males. Female Fmr1 KO rats demonstrated significantly higher freezing rates in response to the conditioned tone compared to wild-type females, but did not show a significant difference in fear conditioning to the trained context. We also note that there is precedent for sexual dimorphism in the fear conditioning test. For example, Maren et al. (1994) reported that male Long-Evans Hooded rats are more responsive to shock-induced fear conditioning than females, while Colon et al. (2018) also showed that...
female Long-Evans rats froze less than males. Thus, in general, wild-type female rats tend to present with less freezing than males in response to fear-conditioning.

In the home cage activity analysis, Fmr1 KO rats of both sexes exhibited an increase in activity compared to the wild-type rats, particularly in the nocturnal dark phase. The apparent discrepancy between the observed hyperactivity during 24-h home cage monitoring and the reduced activity in the open field test, might be explained by the fact that the open field test is an acute experiment conducted over 20 min. in a novel, unfamiliar environment, whereas the home cage activity took place over four days in the familiar surroundings of the home cage. We suggest that Fmr1 KO rats displayed reduced exploratory behavior in the open field arena due to increased anxiety and fearfulness after abruptly being placed in the new environment, whereas the natural hyperactive behavior of the Fmr1 KO rat is unmasked in the more unperturbed situation of the home cage. These findings illustrate that results from two tests, each ostensibly measuring motor activity, can differ greatly in outcomes, likely because of the contexts in which the tests are conducted.

5. Conclusions

Under the conditions delineated here, the Fmr1 KO rat showed reliably measurable differences from the wild-type controls in motor activity, exploratory behavior, social dominance, repetitive behavior, fear conditioning memory, and EEG power spectrum. An important finding of this study was that there are clear differences in the phenotype of male Fmr1 KO rats vs. female KO rats. Marked sex differences in cognitive and clinical profiles have been observed in FXS subjects (Rinehart et al., 2011). We found differences in males vs. female KOs on EEG, the wood chew test of repetitive behaviors where males displayed increased activity while females did not, and on the fear conditioning test where females but not males showed abnormal fear memory.

Male Fmr1 KO rats mimic human FXS males in that most male FXS subjects have no or low FMRP expression. However, female Fmr1 KO rats and mice lacking FMRP differ from most human FXS females who express higher levels of FMRP than males, and in a mosaic fashion in tissues. Therefore, the overall FXS pathology in the human female population is less severe and more variable than that of males with FXS. Additionally, the level of FMRP expression in both males and females has been shown to correlate with the degree of impairment (Budimirovic et al., 2020; Tassone et al., 1999). Nevertheless, despite the higher level of FMRP expression in females vs. males with FXS, Bartholomay et al. (2019) has noted that “females with FXS do not necessarily achieve better outcomes than their male counterparts, and the socio-emotional burden on affected girls, women, and their families may still be equal to that of affected males”. Taken together, our results along with those from other studies on the Fmr1 KO rat, indicate that the Fmr1 KO rat is a useful tool for studying the FXS phenotype, and for testing novel therapies. In this regard, the Fmr1 KO rat model displays many of the core behavioral characteristics and EEG abnormalities of FXS. Given the many physiological, neurological, pharmacological and genetic differences between the rat and the mouse, the two rodent models of FXS could be used in conjunction with each other to conduct more comprehensive translational studies for testing new treatments.

Declaration of Competing Interest

Subba Karumuthil-Melethil, Olivier Danos, and Joseph T. Bruder declare that they own stock in and are employees of REGENXBIO Inc. The other authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2020.105118.

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