ABNORMAL BEHAVIORAL PHENOTYPES IN JUVENILE AND ADULT *Fmr1* KO MICE

By

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Abstract

Fragile X Syndrome (FXS) is the most common genetic cause of intellectual disability with symptoms that overlap with autism spectrum disorders (ASD). FXS is caused by a mutation in the *Fragile X Mental Retardation 1 (Fmr1)* gene and a loss of Fragile X Mental Retardation Protein (FMRP). FMRP is an RNA-binding protein that regulates neuronal function through regulation of protein translation and direct interaction with membrane channels. FXS symptoms include increased anxiety, repetitive behaviors, social communication deficits, delayed language development, abnormal sensory processing, and cognitive deficits. Studies involving FXS patients have demonstrated that there is an increase in the amount of anxiety related activities. To further understand the translational aspect of these behaviors in the mouse model, it is necessary to evaluate different age models such as juveniles and adults. Although there has been extensive research done in the behavior of adult *Fmr1* knockout mice, there is a gap in the literature on juvenile behavioral mouse studies. Filling this gap in knowledge is essential due to the current research addressing children with FXS and having a suitable mouse model will enable a more reliable translational approach. In this study, we will examine the abnormal behavioral phenotypes in juvenile and adult *Fmr1* KO mice by performing both a nest building and nest removal/digging test to determine baseline behavior for the basis of future translational behavioral research.

Acknowledgements

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I. Introduction

Fragile X Syndrome, also known as FXS, is an X-linked dominant neurodevelopmental disorder. Fragile X Syndrome is the most common inherited genetic cause of intellectual disability with symptoms that overlap with autism spectrum disorders (Talisa et al., 2014, Verma et al. 2019). It is estimated that approximately 1 in 6, 000 to 7,143 males and 1 in 8,000 to 11,111 females are affected (Kazdoba et al., 2014, Verma et al. 2019). Because this is an X-linked dominant neurodevelopmental disorder, Fragile X Syndrome symptomatology of males is usually more severe than females with Fragile X Syndrome. This is due to males only possessing one X chromosome while females possess two X chromosomes.

Fragile X Syndrome is caused by a polymorphic region being present at the five prime untranslated end of the non-coding region of the Fragile X Messenger Ribonucleoprotein 1 (*Fmr1*) gene (Verma et al. 2019). This is a mutation that consists of cytosine-guanine-guanine (CGG) trinucleotide repeats in the Fragile X Messenger Ribonucleoprotein 1 gene located in chromosome Xq27.3 (Lozano et al., 2016, Verma et al. 2019, Davidson et al., 2022). In other words, the mutation is located on the X chromosome at the coordinate q27.3. Expansions of more than 200 cytosine-guanine-guanine repeats are referred to as the full mutation and individuals with at least 200 cytosine-guanine-guanine repeats are noted to have Fragile X Syndrome (Schmitt et al., 2022). The mechanism for the mutation is still unknown but thought to occur due to secondary hairpin loop structures (Verma et al. 2019). The secondary hairpin loops cause a disruption in the replication processes allowing for the transcription process to loop itself (Verma et al. 2019). Once stuck in the loop the transcription process goes unchecked allowing for the cytosine-guanine-

guanine repeats to take place (Verma et al. 2019). The cytosine-guanine-guanine repeats cause the gene's function to become altered. This is due to the gene function being majorly disrupted from the cytosine-guanine-guanine repeats leading to epigenetic silencing due to hypermethylation of the cytosine-guanine-guanine repeat region (Verma et al. 2019). Individuals with 55 - 200 cytosine-guanine-guanine repeats are considered a "premutation" (Verma et al. 2019, Schmitt et al., 2022). Premutation carriers may still have similar symptomatology as full mutation individuals, however not as severe (Schmitt et al., 2022). This mutation prevents the proper expression of Fragile X Messenger Ribonucleoprotein (FMRP). (Kazdoba et al., 2014, Verma et al. 2019). The improper expression of the Fragile X Messenger Ribonucleoprotein is due to the heterochromatin formation leading to a microscopically visible constriction on the X chromosome (Verma et al. 2019). Due to the heterochromatin formation the gene becomes silent, making it the Fragile X Messenger Ribonucleoprotein does not form and is unable to properly bind to the ribonucleic acid strand properly. This is due to the Fragile X Messenger Ribonucleoprotein being a ribonucleic acid-binding protein that regulates synaptic function through the regulation of protein translation (Kazdoba et.al, 2014, Verma et al. 2019). Fragile X Messenger Ribonucleoprotein binds to nearly 4% of the neuronal ribonucleic acid by doing so it has a wide control on receptors (Verma et al. 2019). Due to this Fragile X Messenger Ribonucleoprotein is responsible for repression of the activity-dependent group I mGluR neurotransmitter (Verma et al. 2019). When Fragile X Messenger Ribonucleoprotein is not present due to the Fragile X Messenger Ribonucleoprotein 1 gene being mutated mGluR goes unregulated allowing for mTOR activation (Verma et al. 2019). This leads to a cascade of inactivation and phosphorylation ultimately leading to the re-phosphorylation of the Fragile X Messenger Ribonucleoprotein and the repression of translation (Verma et al. 2019). When this cascade remains unchecked due to the

mutated gene this leads to elevated levels of protein synthesis causing the synaptic function to be altered and therefore lead to abnormal behavioral phenotypes (Verma et al. 2019).

It is very commons for individuals with Fragile X Syndrome to exhibit cognitive impairment (Thurman et al., 2022), social and communication deficits (Kaufmann et al., 2004, Verma et al. 2019, Thurman et al., 2022, Schmitt et al., 2020), increased anxiety (Talisa et al., 2014, Oaks et al., 2016, Verma et al. 2019, Protic et al., 2022, McDougle et al., 2022), and sensory hyperexcitability (Miller et al., 1999, Rotschafer et al., 2013, Ethridge et al., 2016, Verma et al. 2019, Razak et al., 2021). Additionally, numerous studies indicate that Fragile X Syndrome individuals exhibit impairments in executive function (Pennington et al., 1996, Schmitt et al., 2019). In particular, a central impairment in Fragile X Syndrome is a deficit in cognitive flexibility (Schmitt et al., 2019, Hooper et al., 2008, Hooper et al., 2018). Cognitive flexibility commonly refers to the ability to adapt to changing environmental demands by switching to a different or new choice pattern (Ragozzino et al., 2007). This ability is critical for successful daily living. While Fragile X Syndrome individuals commonly exhibit deficits in cognitive flexibility, it is unclear which cognitive and brain processes underlie the cognitive flexibility impairments. This is critical to understand as preclinical studies indicate that cognitive flexibility deficits may arise for different reasons (Amodeo et al., 2012, Kim et al., 2005, Baker et al., 2014, Ragozzino et al., 1999, Harrop et al., 2021) e.g. inability to initially inhibit a previously learned strategy (perseveration) vs. inability to inhibit from choosing irrelevant choices. Both the biochemical and behavioral phenotypes have been found to be the same in both mice and humans allowing for the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice to be a good translational model (Verma et al. 2019). However, even though there are parallels in how Fragile X Syndrome affects both mice

and humans who possess the genetic mutation the need for biomarkers to be established is necessary for accurate translational models to be established. A recent NIH workshop on biomarkers in neurodevelopmental disorders (Sahin et al., 2018) identified the urgent need to identify translation-relevant biomarkers in preclinical models in conjunction with clinical work. In this study, our goal was to identify translation-relevant behaviors in a preclinical mouse model of Fragile X Syndrome.

To understand the translational aspect of these behaviors in a mouse model, it is necessary to evaluate the onset and development of Fragile X Syndrome behavioral phenotypes at various time points, such as in juvenile and adult animals. There has been extensive research conducted on the behavior of adult Fragile X Messenger Ribonucleoprotein 1 gene knockout mice such as novel item recognition, social interaction, fear conditioning, marble burying, elevated plus maze, and open field testing to name a few (Pirbhoy et al., 2021, Reyes et al., 2021, Reinhard et al., 2019, Cogram et al., 2019, Gurney et al., 2017, Melancia et al., 2018, Spencer et al., 2005). Despite the abundance of Fragile X Messenger Ribonucleoprotein 1 gene knockout mouse behavioral assessments, there is still a gap in the literature on juvenile behavioral mouse studies (Spencer et al., 2005, McNaughton et al., 2008, Spencer et al., 2008, Liu & Smith 2009). Findings in juvenile mice, such as postnatal day 21 (P21), can be compared and correlated to findings in human children (Dutta et al., 2016). The increase in investigations conducted on children with Fragile X Syndrome further emphasizes the need to fill this gap in knowledge through the creation of a suitable animal model to study translationally relevant aspects of Fragile X Syndrome pathology. To help bridge this gap, we developed a baseline assessment of juvenile Fragile X Messenger Ribonucleoprotein 1 gene knockout behavior based on the current adult animal model.

This assessment enabled the collection of baseline behavioral data for juvenile mice that (A) lent to the establishment of a translationally relevant animal model of childhood Fragile X Syndrome, and (B) compared Fragile X Syndrome development and progression in juvenile and adult animals across age-specific models. Nesting removal (digging) and nest building assays were used in this study to test cognitive flexibility ("sameness") and assess successful daily living respectively. Individuals with Fragile X Syndrome and autism spectrum disorders generally exhibit a strong desire to maintain a specific routine or same environment (Nicolaidis et al., 2014, Prior et al., 1973). A change in routine or environment can be distressing and cause repetitive behaviors (Nicolaidis et al., 2014). Rodents show an innate preference to create nests as it is important for heat conservation, reproduction, and to provide shelter from predators.

II. Methods

Animals

Homozygous female Fragile X Messenger Ribonucleoprotein 1 gene knockout mice and hemizygous male Fragile X Messenger Ribonucleoprotein 1 gene knockout mice on a C57BL/6 background (B6.129P2-Fragile X Messenger Ribonucleoprotein 1 gene^{tm1Cgr}/J, stock #003025) (Bakker et al., 1994) along with male and female and C57BL/6J wild type controls (stock #000664) were obtained from Jackson Laboratories. Fragile X Messenger Ribonucleoprotein 1 gene knockout mice were mated to generate experimental mice and C57BL/6J wild type mice were mated together to generate control mice. All genotypes were confirmed by Transnetyx (Cordova, TN) using real-time PCR analysis (Fragile X Messenger Ribonucleoprotein 1 gene knockout probes: Forward Primer: GCTAAGAATGACTTGTTTATTTCTCTCAGTTTT, Reverse Primer:

GGCTTCTGAGGCGGAAAGA, Reporter 1: ATGTGATGCTACTAGAGCCCCA; Fragile X Messenger Ribonucleoprotein 1 gene wild type probes: Forward Primer: CAGTTTTATGTGATAGAATATGCAGCATGTG, Reverse Primer: ACAGATCGTAG ACGCTCAATTGTG, Reporter 1: ATGCTACGTATAATGA- AATTGT). Mice in this study were weaned between 18 to 21 days of age and were grouped housed (maximum of 5 animals per cage) until initiation of experiments. All animals were housed and maintained in an AAALAC-accredited vivarium facility under a 12-hour light/dark cycle (7 am/7 pm) and were provided an irradiated rodent diet (PicoLab, 5053) and water ad libitum for consumption.

All procedures in this study were performed with the approval from the University of California Institutional Animal Care and Use Committee (IACUC) and in accordance with the NIH Animal Care and Use Guidelines. Male mice were used in this study. 15 eight-week-old Fragile X Messenger Ribonucleoprotein 1 gene knockout, 15 eight-week-old wild type, 15 P21 Fragile X Messenger Ribonucleoprotein 1 gene knockout and 15 wild type mice (a total of 60 mice) were used. For the purpose of this study, the P21 age group refers to newly weaned pups ranging from 21 to 22 days of age at the behavioral assessment testing. All behavior testing was performed at a similar time in the morning each day and by the same experimenter blinded to genotype.

Nest removal (digging) Assay

Three days prior to nest removal (digging) testing, animals were individually housed in a cage with bedding and nesting material (a pressed cotton square nestlet), which allowed the animals sufficient time to shred their nestlet and make a nest. On the morning of the third day, animals were transferred in their home cage to the behavior room one hour before the initiation of testing

to allow for acclimation to the room. A maximum of 6 mice were tested at one time. Cage wire feeders, water bottles and lids were removed from the cage to prevent distraction and for a clear view of digging. A clear plastic sheet was placed over the cage to prevent the mice from crawling out of the cage. Mice were placed directly under a camera to record activity during the testing session. Nesting removal behavior was measured over the course of two 10-minute video recorded sessions by documenting the total time spent digging in their home cages. Time spent digging in the bedding was measured with undisturbed nesting material in the home cage (10 minute with nest) and subsequently with the nesting material removed from the home cage (10 minute without nest) (Lacivita et al., 2020). Testing video sessions were reviewed, and a stopwatch was used to record the total amount of time spent digging in seconds. Digging was classified as any movement in which the mouse used its nose, forelimbs, and/or hindlimbs to displace the bedding of the cage. The same parameters were used for adult and juvenile animals; however, the juvenile mice were weaned at P18-19 and the digging test was performed at P21-P22. After data collection, nests were placed back into the cage of the animal and animals were provided fruity cereal as a reward for completing the task. Animals were transferred back to the vivarium facility and provided with extra environmental enrichment since they were individually housed.

Nest Building Behavior Assay

Behavioral testing parameters remained the same for the nest removal digging test in the adult and juvenile mice, however adjustments to our nest building behavior protocol were necessary to accommodate the juvenile mice. A smaller nestlet size (weight) was used for the nest building behavior test, however all other parameters remained the same. Nest building behavior was measured in the home cage of the animal following an overnight period of 14 hours total. Animals

were individually housed and were placed in new clean cages. Animals were transferred to the behavior room one hour before the initiation of testing (5 pm) to allow for acclimation to the room. A maximum of 6 mice were tested in the room at one time. Pre-weighed nestlets, ranging from 2.400 - 2.600 g were used for adult mice while pre-weighed nestlets, ranging from 0.5000 - 0.5500 g were used for juvenile mice. Nestlets were placed in the cages at 6 pm (1 hour before the dark cycle began). Confirmation that lights were off at 7 pm occurred for each cohort tested. The following morning, at 8 am (1 hour after the dark cycle ended, i.e., lights on) the nest building behavior was scored and an image of the nest was taken for reference. The largest piece of untorn nestlet was weighed and the percent of intact nestlet weight was calculated. Alternatively, all intact pieces can be weighed for the percent of intact nestlet. After data collection, nestlets were placed back into the cage of the animal and animals were provided fruity cereal as a reward for completing the task. Animals were transferred back to the vivarium facility and provided with extra environmental enrichment since they were individually housed.

The 5-point scale was used to score nest building: 1. The nestlet is largely untouched (>90% intact), 2. The nestlet is partially torn (50-90% intact), 3. The nestlet is mostly shredded and less than 50% of the nestlet remains intact, but less than 90% is within a quarter of the cage floor area, 4. An identifiable but flat nest: more than 90% of the nestlet is torn, the material is gathered into a nest within a quarter of the cage floor, but the nest is flat, with walls higher than the mouse body height (curled up on its side) on less than 50% of its circumference, 5. A near-perfect nest: >90% of the nestlet is torn, the nest is a crater, with walls higher than mouse body height on more than 50% of its circumference (Deacon 2006). A near-perfect nest is not as common as the other scores.

To measure the amount of nestlet building using the percent of nestlet left intact, the equation used to calculate the percent intact is:

% intact=W2 (g)W1 (g) x 100

Where W2 is the largest piece of nestlet that was left intact and W1 is the original weight of the nestlet. The results of this calculation determined the degree of nest building during the testing session and if there were significant nest building differences between the genotypes for each age group.

Data Analysis

Statistical analysis was performed using the program GraphPad Prism. Data blinded and analyzed for genotype (wild type, Fragile X Messenger Ribonucleoprotein 1 gene knockout). A Two-way ANOVA test allowed for the different genotypes in each group to be compared on the same scale. A power analysis determined the sample size needed to obtain significance was conducted. Calculations were performed, setting alpha level to 0.05, the power to 0.80, and the effect size was set to approximate a difference in means based on previous studies (Cogram et al., 2020; Lacivita et al., 2020).

III. Results

Nest removal digging assay

A two-way ANOVA test was conducted to determine if the difference in the nesting condition significantly contributed to a change in digging time. For the adult mice there was no significant difference between the wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout

digging times when the mice were tested with their undisturbed nest (with nest) **(Figure 1).** There was also no significant difference between the juvenile wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout digging times when the mice were tested with their undisturbed nest (with nest) **(Figure 2).** There was, however, a significance in the amount of digging time between the wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout in the adult mice with the nest removed (without nest) **(Figure 3)**, and there was significance difference in digging time of the P21 Fragile X Messenger Ribonucleoprotein 1 gene knockout mice when the nest was removed as well. **(Figure 4)**. In both age groups, Fragile X Messenger Ribonucleoprotein 1 gene knockout mice displayed a significantly increased digging time compared to their wild type counterpart. These results show that Fragile X Messenger Ribonucleoprotein 1 gene knockout mice of both age groups exhibit a decrease in both the formation of a nest and the use of the nestlet compared to the wild type mice. This suggests that by causing a disturbance to their environment, a significant anxiety response is produced as a result of this change.

Nest Building Behavior Assay

A two-way ANOVA test was used to determine if there was a significant difference between the wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in their nest building behavior. Both the nestlet score and percentage of nestlet intact were analyzed in this study. Adult Fragile X Messenger Ribonucleoprotein 1 gene knockout mice displayed significantly decreased nest building scores compared to wild type mice **(Figure 5)**. P21 Fragile X Messenger Ribonucleoprotein 1 gene knockout mice also displayed significantly decreased nest building scores compared to their wild type counterpart **(Figure 6)**. When comparing the wild type and

Fragile X Messenger Ribonucleoprotein 1 gene knockout adult mice, there was a significant difference in the percent of nestlet intact as well **(Figure 7)**. There was also a significant difference in the percent of nestlet intact for the Fragile X Messenger Ribonucleoprotein 1 gene knockout P21 mice **(Figure 8)**. Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in both age groups showed a higher percent of nestlet left intact compared to wild type mice. These results show that Fragile X Messenger Ribonucleoprotein 1 gene knockout mice of both age groups exhibit a decrease in both the formation of a nest and use of the nestlet compared to the wild type mice.

IV. Discussion

In this study, we evaluated both the nest removal digging and nest building behavior of adult and juvenile (P21) Fragile X Messenger Ribonucleoprotein 1 gene knockout and wild type animals. We have confirmed that the behavioral findings of juvenile animals correlated with those of adult animals (Deacon 2016, Lacivita et al., 2020). This led to the establishment of an entirely new Fragile X Syndrome juvenile behavioral model to use in preclinical translational research. First, Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in both adult and juvenile age groups were found to have an increased digging time after the removal of the nest from their cage. There was no significant difference in the digging time with the nest, however, that was expected as there is no change in the environment. The nests remained in the cages, thus an anxiety-related behavior such as increased digging was not exhibited in the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice. Second, Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in both adult and juvenile age groups were found to have an increased percent of nestlet left intact. Third, Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in both

adult and juvenile age groups were found to have a decreased nestlet score. Overall, the nest building 5-point score can be viewed more as a subjective test as the score may vary between experimenters. For this reason, the weights of the intact nestlets were used for a more objective measure of nest building behavior to determine the percent of nestlet intact as a proxy for the nest building test. Both tests yielded robust significant differences between the wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout mice, therefore either test can be used for nest building behavioral assessment.

To date, there is only one study that performed the nest removal digging behavior assay and a protocol for adult mice has been established at length (Lacivita et al., 2020), however no such protocol has been established for juvenile animals—namely P21 mice. We aimed to validate this adult protocol and modify the protocol for juvenile mice. As a result, we created a new baseline behavioral model that demonstrates there are robust and significant behavioral differences between the wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout mice **(Figures 3-4)**. This reinforces the knowledge that the lack of production of Fragile X Messenger Ribonucleoprotein 1 gene protein leads to a sensory processing deficit. The correlation between the increase in digging time and sensory processing deficit is due to digging being representative of an induced anxiety state. An increase in anxiety is due to the removal/change in their environment. Since the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice exhibited increased digging when their nestlets were removed, it demonstrates that the mice were unable to compensate for the change in their environment, thus the need for "sameness". The change in the environment acts as a sensory stimulus/anxiety inducer. When the sensory stimulus is then paired with the lack of recovery/longer digging times it reinforces the notion that the removal of the

Fragile X Messenger Ribonucleoprotein 1 gene promotes high anxiety and sensory processing deficits. In turn, this is visualized by the data which showed an increased anxiety specifically when their environment has been altered from their normal due to the increased digging times since mice dig when anxious **(Figures 3-4)** (Lacivita et al., 2020). Our results show that there is a significant increase in the amount of time Fragile X Messenger Ribonucleoprotein 1 gene knockout mice spend digging in both age groups signifying that the behavioral phenotype is constant across the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice regardless of age group. However, we are the first to show that this robust significant increase is present in the juvenile Fragile X Messenger Ribonucleoprotein 1 gene knockout mice **(Figure 4).**

Similar to the nest removal digging assay, there are no current studies that incorporate the use of juvenile mice in nesting building behavioral studies. By modifying the adult protocol to alter the nestlet size to an appropriate size for P21 mice, we were able to create a baseline for nesting building behavior. In both age groups there was a significant difference between the wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in both the nest building score and percent of nestlet intact **(Figures 5-8)**. However, the difference in nesting building behavior has never been recorded in juvenile mice until now. The significant decrease in the nestlet score for the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice compared to the wild type mice indicates that the abnormal sensory processing inFragile X Syndrome is present **(Figure 6)**. This demonstrates that the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice have a decreased motivation to build nests. Nest building is an innate behavior in rodents that help to provide comfort and security. Since the Fragile X Messenger Ribonucleoprotein 1 gene knockout mice lack the motivation to build a nest, it also demonstrates that the social deficit impedes the

natural instinct to build a nest (Deacon 2006, Spencer et al., 2008). The significant increase in percent of nestlet remaining intact that the juvenile Fragile X Messenger Ribonucleoprotein 1 gene knockout mice exhibit also reinforces the presence of a sensory processing deficit known to be found in Fragile X Syndrome, even at an early age of development **(Figure 8)** (Gilotty et al., 2002, McDuffie et al., 2010). This trend has been observed in the adult Fragile X Messenger Ribonucleoprotein 1 gene knockout mice; however we are the first to show this in juvenile mice (Deacon 2006, Spencer et al., 2011). This is groundbreaking and will hopefully advance preclinical research.

In summary, the behaviors of nest removal and nest building in juvenile Fragile X Messenger Ribonucleoprotein 1 gene knockout mice have not been examined until now. By finding this significant difference between juvenile wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in digging and nest building behaviors, we have established a baseline for future studies. The P21 mice model the adolescent age group more closely than adult mice (Dutta et al., 2016). Our data demonstrate that juvenile Fragile X Messenger Ribonucleoprotein 1 gene knockout mice show the same significant difference in behavioral phenotypes as the Fragile X Messenger Ribonucleoprotein 1 gene knockout adult mice. Based on the development of this new juvenile behavior model of Fragile X Syndrome, future studies can be done to determine the effects of pharmacological interventions targeting children with this neurological condition. Identification of comparable biomarkers in humans and validated animal models is a critical step in facilitating preclinical to clinical therapeutic pipelines to treat neurodevelopmental disorders (Ethridge et al., 2019, Berry-Kravis et al., 2018, Jonak et al., 2020).

Individuals with Fragile X Syndrome and autism spectrum disorders experience impaired processing of sensory stimuli. There is no FDA-approved treatment available to treat the underlying cause of Fragile X Syndrome. Currently, families rely on behavioral therapies or off-label medications to treat symptoms, which are of marginal benefit with deleterious side effects (Tranfaglia et al., 2018). There is an unmet need to develop translational and objective electrophysiological biomarkers in mouse models of Fragile X Syndrome that match human electroencephalography biomarkers and behavioral alterations (Berry-Kravis et al., 2018, Jonak et al., 2020, Jonak et al., 2021, Ewen et al., 2019, Lovelace et al., 2018). Examining a range specific drug–behavior phenotypes and the use of electroencephalography in both species with multiple drugs will also allow for specific drug–measure predictions and data-guided stratification of patient populations for clinical trials. The data from this study may also directly address another major issue that can impact clinical trials – development of tolerance to drugs. The comparison of acute and chronic treatment experiments can use a longitudinal approach to test immediate effects on specific behavioral measures (acute) and how the effect differs after chronic treatment. Further, different pharmacological treatments have been shown to improve cognitive flexibility by affecting distinct processes (Baker et al., 2014, Amodea et al., 2014, Brown et al., 2012, Mohler et al., 2012, Ragozzino et al., 2012). Thus, identifying distinct behaviors with electroencephalography biomarkers in Fragile X Syndrome individuals and how they relate to unique alterations in multi-regional electroencephalography patterns may allow the development of successful, individualized treatments in reducing a core impairment in Fragile X Syndrome (Berry-Kravis et al., 2013, Hagerman et al., 2009, Henneberry et al., 2021). Individualized treatments may be necessary due to the wide variance and degree of symptoms in each patient.

Some patients respond well to a specific pharmacological treatment intervention, while other patients do not respond to treatment. This phenomenon has been quite a challenge in clinical trials.

The scientific power, yielding the most robust results, is in conducting electroencephalography testing during behavioral tasks to determine the interactions of specific electroencephalography changes during behavioral assessment and tasks (Pennartz et al., 2011). However, in this study non-electroencephalography implanted mice were tested for simplicity and reproducibility. In future studies, we aim to validate behavioral paradigms and differences between electroencephalography-implanted freely behaving wild type and Fragile X Messenger Ribonucleoprotein 1 gene knockout mice in order to ultimately combine electroencephalography and behavior in individual mice. Such studies would uniquely enable correlations between particular drug-induced electroencephalography alterations and behavioral outcomes in individual mice. Go/no-go decisions on further testing of a particular drug would be based upon the magnitude of its effects on electroencephalography and behavior, and then commensurate effort would be made to determine the electroencephalography correlates of profound behavioral improvements. This novel approach will ultimately lead to further advancements in the treatment of Fragile X Syndrome symptomatology in both adults and children.

V. Figures

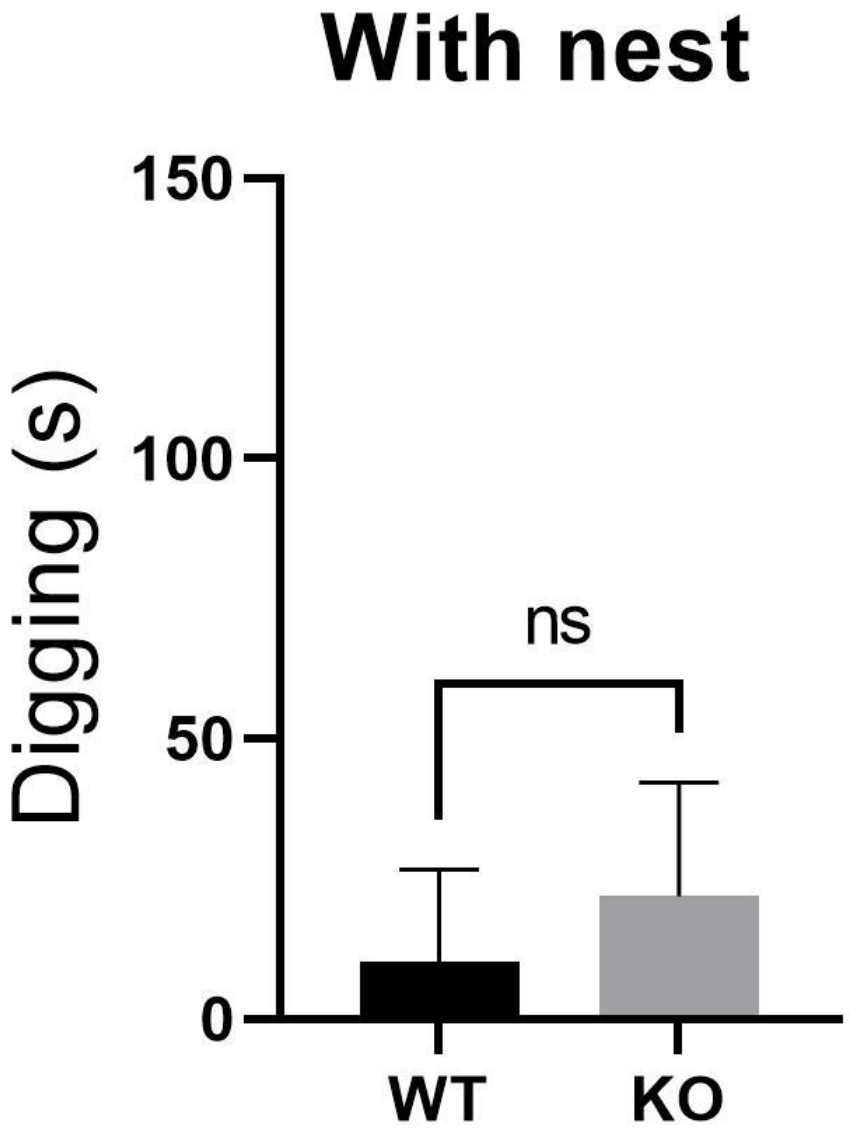

Figure 1. Total time spent digging by adult WT vs. Fragile X Messenger Ribonucleoprotein 1 gene KO mice during a 10 min session with nest. The was no difference between the WT and Fragile X Messenger Ribonucleoprotein 1 gene KO digging time when the nestlet was left in the cage. n.s = no significance. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

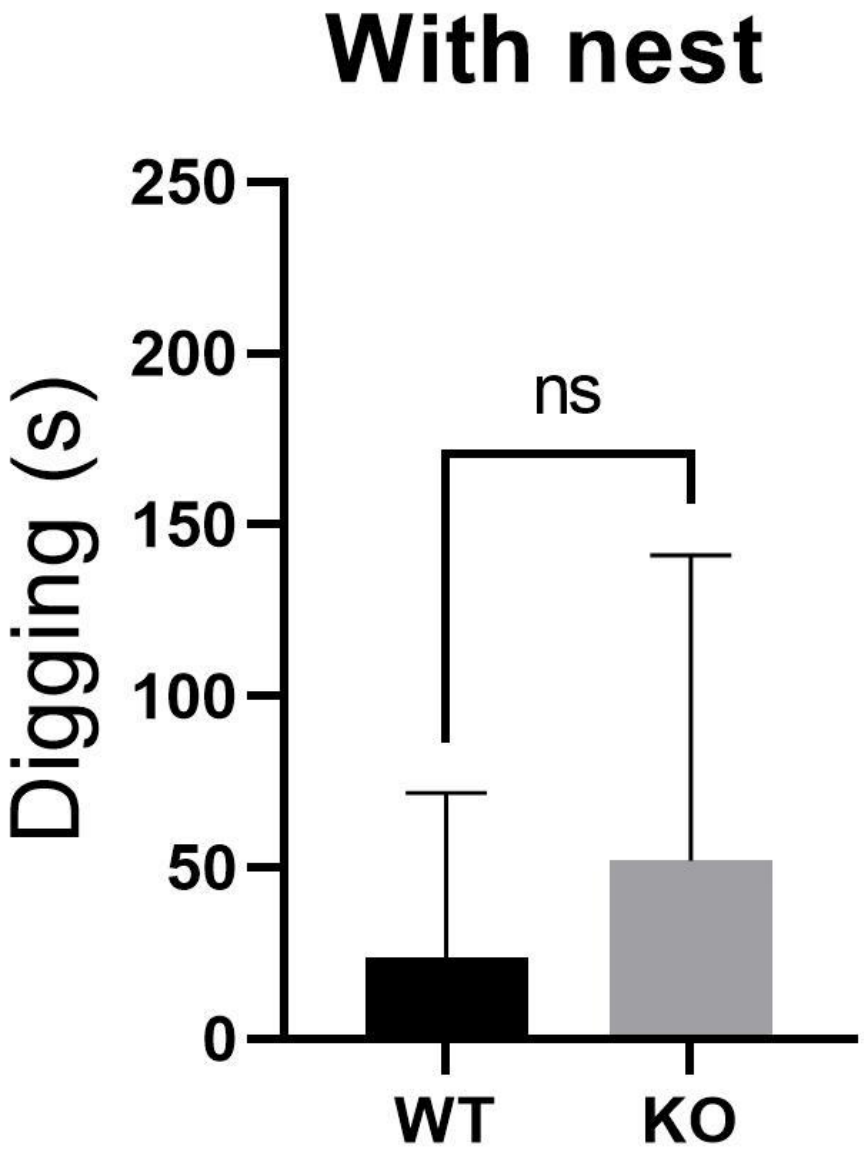

Figure 2. Total time spent digging by P21 WT vs Fragile X Messenger Ribonucleoprotein 1 gene KO mice during a 10 min session with nest. The was no difference between the WT and Fragile X Messenger Ribonucleoprotein 1 gene KO digging time when the nestlet was left in the cage. n.s = no significance. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

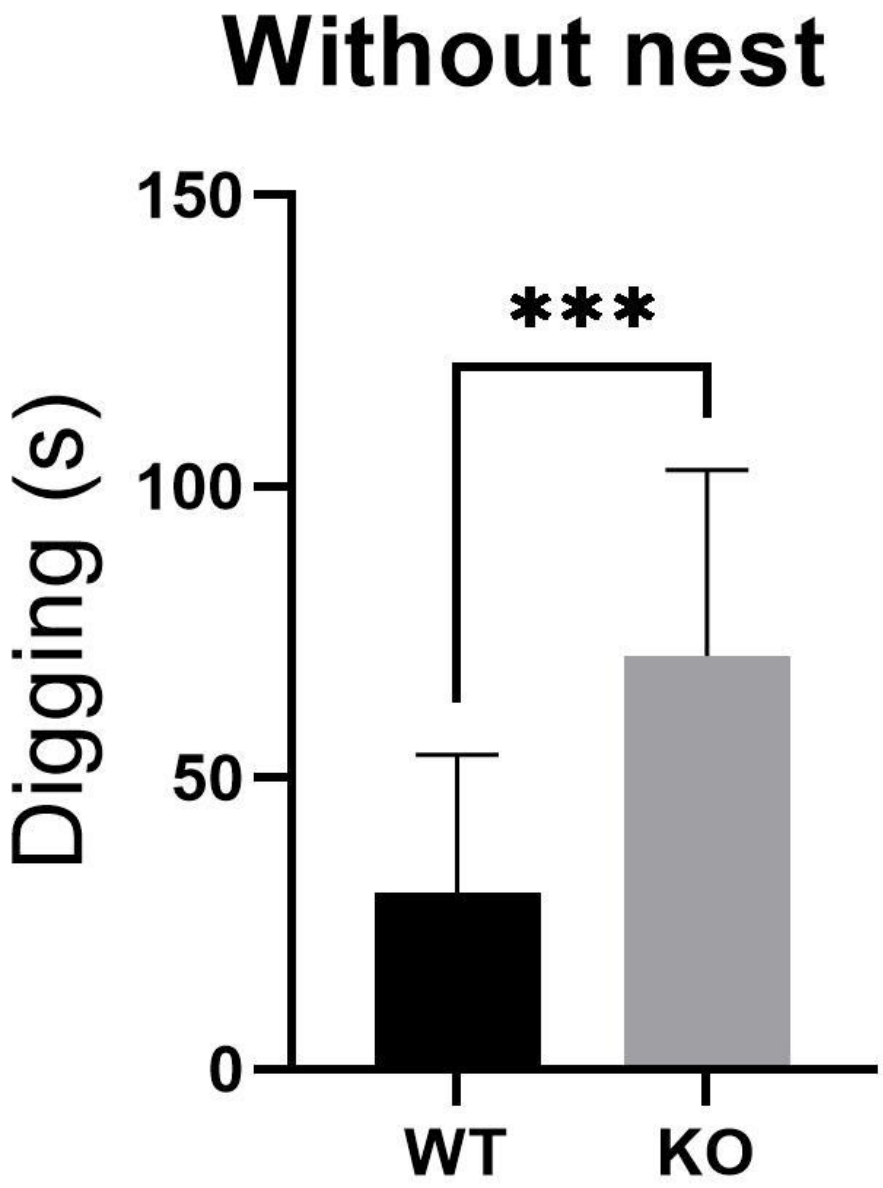

Figure 3. Total time spent digging by adult WT vs Fragile X Messenger Ribonucleoprotein 1 gene KO mice during a 10 min session without nest. Adult Fragile X Messenger Ribonucleoprotein 1 gene KO mice displayed a significantly greater amount of time digging compared to WT mice. *p < .05; **p < .01, ***p < 0.001. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

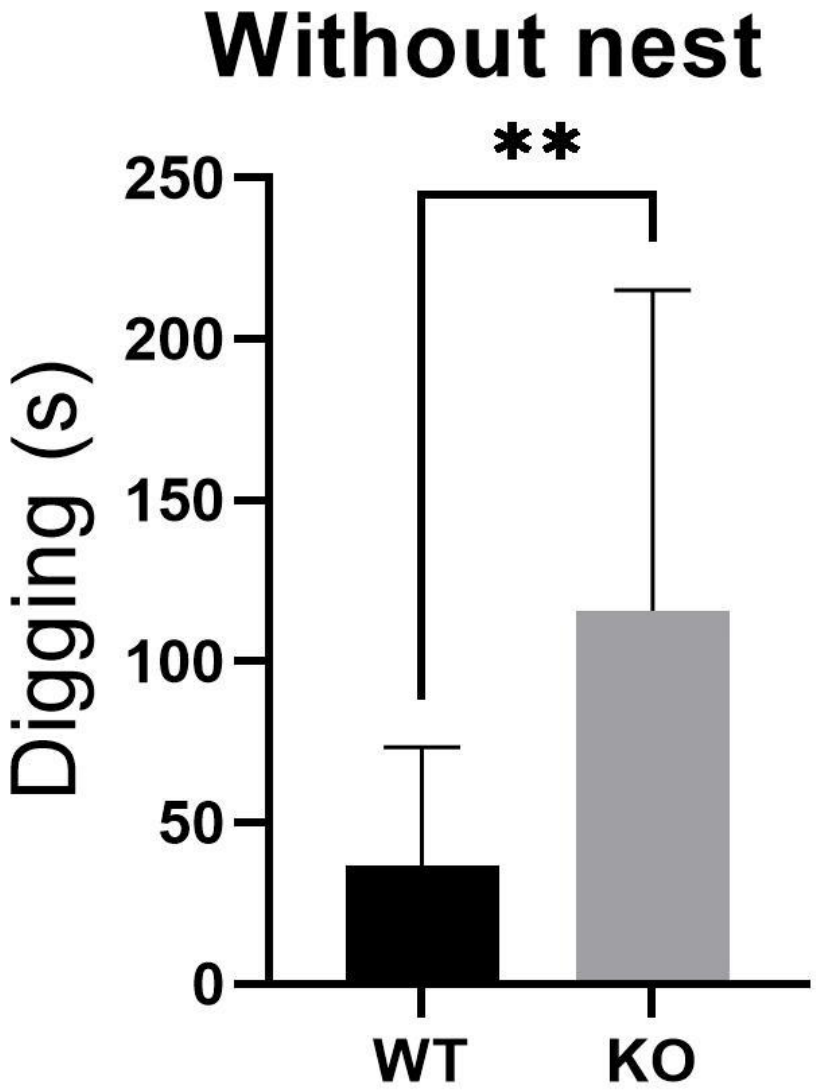

Figure 4: Total time spent digging by P21 WT vs Fragile X Messenger Ribonucleoprotein 1 gene KO mice during a 10 min session without nestlet. P21 Fragile X Messenger Ribonucleoprotein 1 gene KO mice displayed a significantly greater amount of time digging compared to WT mice. *p < .05; **p < .01, ***p < 0.001. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

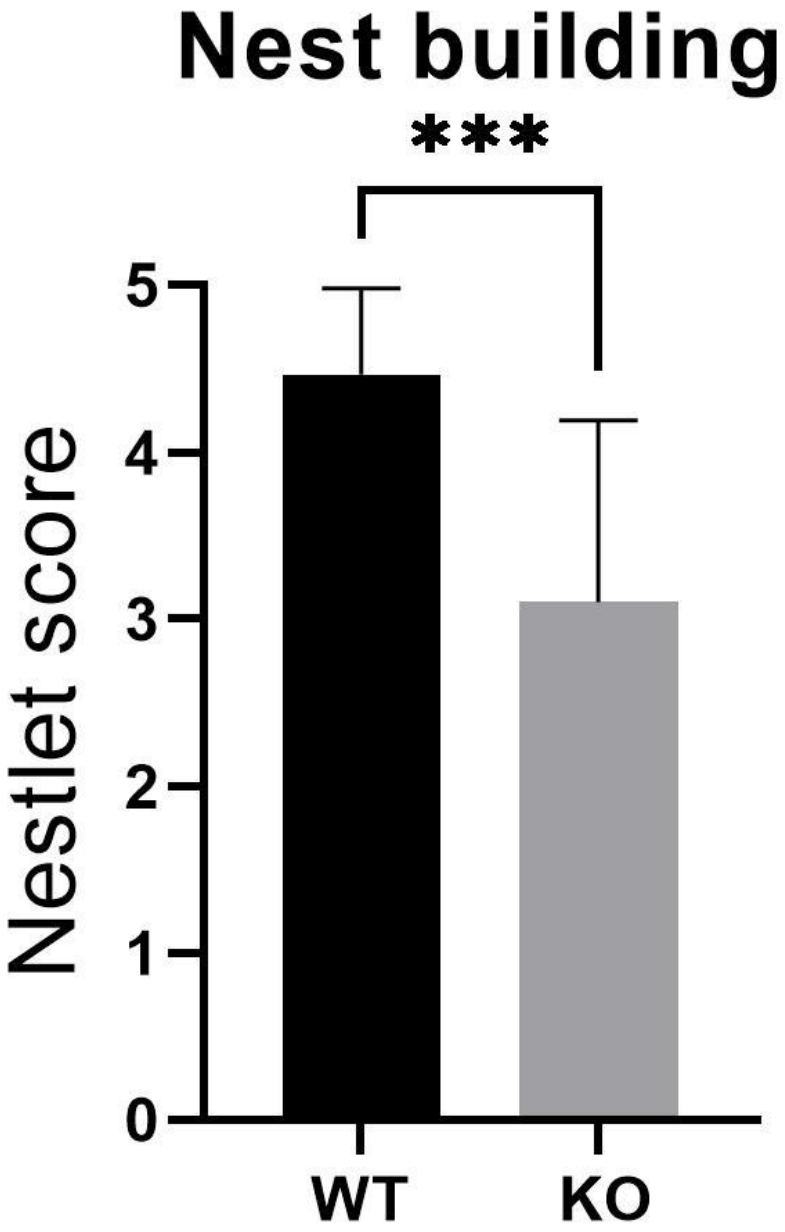

Figure 5. Nest building score for adult WT vs Fragile X Messenger Ribonucleoprotein 1 gene KO mice. Adult WT mice displayed a significantly greater nest building score compared to Fragile X Messenger Ribonucleoprotein 1 gene KO mice. *p < .05; **p < .01, ***p < 0.001. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

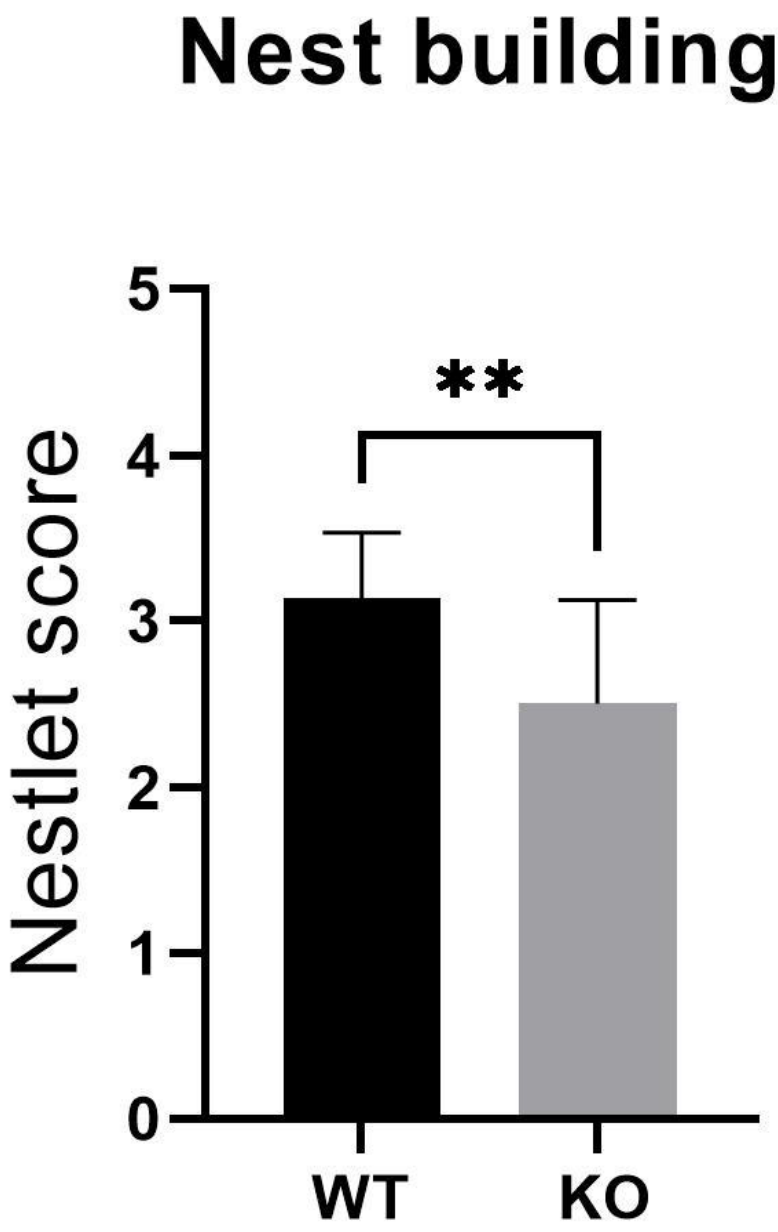

Figure 6. Nest building score for P21 WT vs Fragile X Messenger Ribonucleoprotein 1 gene KO mice. P21 WT mice displayed a significantly greater nest building score compared to Fragile X Messenger Ribonucleoprotein 1 gene KO mice. *p < .05; **p < .01, ***p < 0.001. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

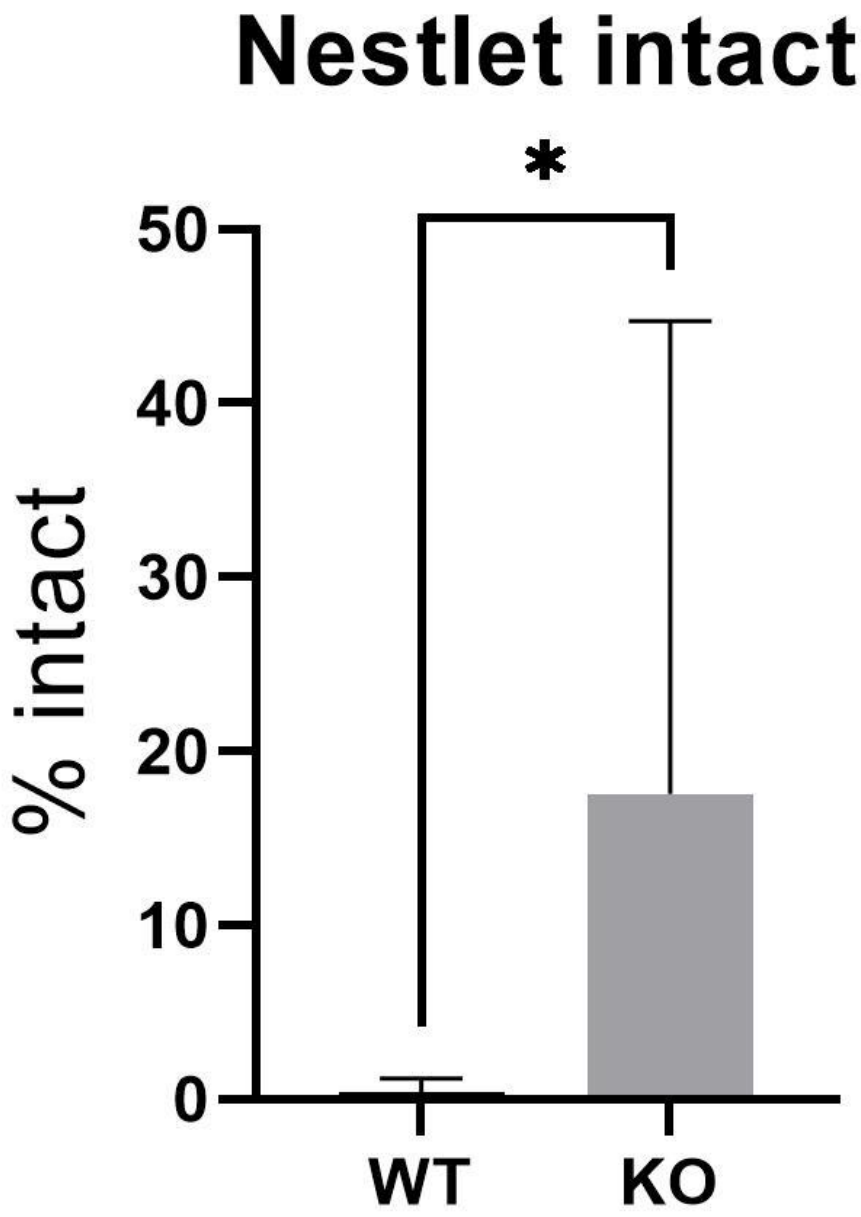

Figure 7. Percent of nestlet intact for adult WT vs Fragile X Messenger Ribonucleoprotein 1 gene mice. Adult Fragile X Messenger Ribonucleoprotein 1 gene KO mice left a significantly greater percentage of their nestlet intact compared to WT mice. *p < .05; **p < .01, ***p < 0.001. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15.

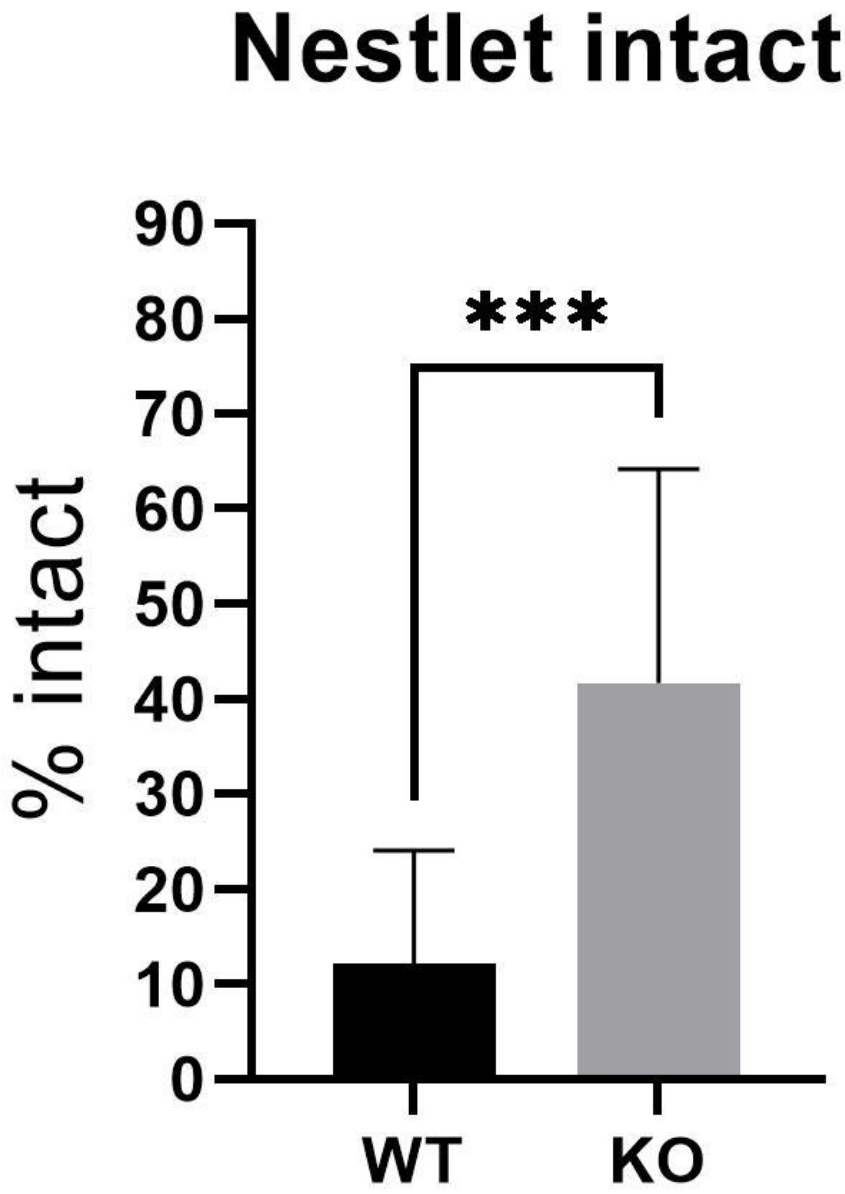

Figure 8: Percent of nestlet intact for P21 WT vs Fragile X Messenger Ribonucleoprotein 1 gene KO mice. P21 Fragile X Messenger Ribonucleoprotein 1 gene KO mice left a significantly greater percentage of their nestlet intact compared to WT mice. *p < .05; **p < .01, ***p < 0.001. n = 15 WT, n = Fragile X Messenger Ribonucleoprotein 1 gene KO 15

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