Exploring the Dynamics of Social Interactions During the Juvenile Stage in a Mouse Model of Fragile X Syndrome

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Abstract

Introduction: Fragile X Syndrome (FXS), caused by mutations in the *Fmr1* gene, is a neurodevelopmental condition linked to cognitive and behavioral differences, including atypical interactions and heightened anxiety-like responses in social settings. Previous studies that used FXS mouse models focused mostly on adulthood but not on behaviors displayed by juvenile mice. This is especially concerning considering the extensive body of research conducted in children and adolescents with FXS, which indicates dynamic neurodevelopmental changes from childhood to adulthood. Thus, it is crucial to study how FXS affects social interactions during the juvenile stage, in both female and male mice. In this study, we compared the social behavior of *Fmr1* knockout (KO) mice, a model of FXS, with wild-type (WT) mice of the C57BL/6J strain.

Methods: We examined both male and female juvenile mice at postnatal day 40 (P40). Social interaction tests were carried out using two mice of the same sex and genotype, who previously had not encountered each other, and were allowed to interact freely in an empty chamber for 10 minutes. Their behaviors were recorded by an overhead camera for 10 minutes. Key social behaviors such as head, body, anogenital sniffing, and physical touch, as well as distance traveled, were analyzed with a recently developed marker-less tracking software.

Results: Results from the social interaction tests showed pronounced sex and genotype differences between juvenile females and males of both the WT and KO genotypes. Overall, sex differences were found in all the behavioral parameters that were measured. Notably, the KO females engaged in more social interaction events compared to WT females in each social behavior. On the other hand, there were no significant differences observed between the WT and KO males.

Discussion: These results highlight the importance of considering the differences that arise during the juvenile stage in *Fmr1* KO mouse model, suggesting that early post-natal phases may be a promising window for therapeutic interventions.

Keywords: Fragile X syndrome, autism, mouse model, social interaction, sex difference, adolescence, neurodevelopment

List of Abbreviations Used (if any):
FXS: Fragile X Syndrome
Fmr1: Fragile X Messenger Ribonucleoprotein 1
FMRP: Fragile X Messenger Ribonucleoprotein 1
ADHD: Attention deficit hyperactivity disorder
ASD: Autism spectrum disorder
KO: Knockout
WT: Wildtype

Introduction

Fragile X syndrome (FXS) is an X-linked neurodevelopmental condition.[1] It results from a triplet CGG expansion within the 5'-untranslated region of the Fragile X Messenger Ribonucleoprotein 1 gene (*Fmr1*), leading to the loss of encoding of the Fragile X Messenger Ribonucleoprotein (FMRP).[1] It is the leading monogenic cause of autism, as autism spectrum disorder (ASD) is present in around 50-70% of individuals with FXS.[2] The FMRP protein plays a fundamental role in facilitating the normal development of the nervous system through the expression of key molecules involved in receptor signaling.[3] The expansion of the triplet sequence causes the development of various FXS-related phenotypes.[4] FXS is linked to cognitive and behavioral differences in humans, including hyperactivity, heightened anxiety-like responses in social settings, and decreased brain activation in brain areas such as the amygdala that mediate social cognition.[5,6]

Social behavior is a broad term that encompasses any form of communication or interaction between two or more individuals.[7] Expressing dynamic social behavior is necessary for adapting to new environments, and it can be expressed in various ways, such as aggression or cooperation.[7] Engaging in social behavior involves continuously recognizing cues that form an individual's response according to how their environment is perceived.[7] Further, social interactions are intricately tied to an individual's emotional state, and pose significant challenges for research into the underlying mechanisms.[7] Though some evidence indicates that individuals with FXS display a genuine interest in social interaction with their peers, they often experience anxiety and consequently exhibit social avoidance when encountering unfamiliar people in foreign environments.[8] This has been illustrated in the greeting behavior of males with FXS who tend to avoid eye contact and physically orient their bodies away from the person approaching them.[9] High occurrence of intellectual disability in FXS, such as attention or working memory deficits, contribute to difficulties in social communication.[9]

Difficulties with social interactions are common in FXS, especially during development. There are higher rates of total behavior problems, such as social withdrawal and anxiety, found in both children and adolescents than in adults.[1] In fact, approximately 81% of males with FXS displayed social avoidance from ages 4 months to 25 years, which emerged during infancy and increased in severity across childhood before stabilizing

through adolescence and early adulthood.[10] This often hinders their ability to demonstrate typical social engagement.[10] Specifically, findings from a study by Black et al. suggests that both behavioral and physiological signs of social anxiety were evident at 12 months of age in infants with FXS.[11] Another study found that adaptive behaviors in a socially favorable manner developed more slowly in young boys with FXS aged between 36 and 95 months.[12] Other studies have found that children with FXS displayed significantly higher levels of social avoidance compared to typically developing children.[13] These behaviors manifest through increased anxiety, gaze aversion, and social avoidance during a socially challenging task.[9] The highest prevalence of clinical concern for individuals with FXS aged between 3 and 30 years was associated with social issues, anxiety, and adaptive social problems.[14]

Though the FXS phenotype affects both males and females, it has been reported that males tend to show more pronounced cognitive impairments compared to females.[15] However, the mechanism behind such differences remains unclear. Recent estimates of the prevalence of FXS in males and females are 1 in 4,000 males and 1 in 8,000 females.[16] This discrepancy is speculated to be due to the presence of an extra X chromosome in females, which compensates for the affected chromosome.[15] Although there are striking differences in the severity and prevalence of the FXS phenotype between males and females, research so far on FXS has predominantly centered around males. However, there is a growing recognition of sex distinctions in social interactions observed in FXS, and the necessity to closely examine social behaviors and their manifestations in the female populations. FXS phenotypes in females tend to manifest as learning disabilities, behavioral problems, shyness, anxiety, and depression.[2] Regarding social skills, girls with FXS between the ages of 7 and 18 years displayed significantly lower rates in social information processing tasks, such as identifying the causes and consequences of social problems.[17] In a recent study by Lightbody et. al., levels of depressive mood symptoms and social avoidance worsened in female patients with FXS as they aged.[18] These symptoms exhibit a direct correlation with social communication, reciprocal social behavior, and adaptive function in female patients with FXS.[18] These qualities can have a substantial impact on their overall quality of life. Since affected males are capable of passing the full mutation to their daughters, investigation of heterozygous females through behavioral analysis is highly important.[19] Because of their less severe cognitive deficits, FXS female carriers are more suitable for behavioral studies as their phenotypic deficits are not masked by the other developmental causes seen in FXS males with the full mutation.[19]

Several studies have utilized the *Fmr1* knockout (KO) mouse model of FXS to investigate social behaviors. This is because they exhibit certain similar traits in the human syndrome, such as hyperactivity, intellectual and cognitive differences, as well as reduced preference for novelty in social settings.[20,21] Spencer et al. showed that social behaviors of male *Fmr1* KO mice at 3-4 months of age are dependent on experience and KO mice show more social behavior with familiar mice.[22] Additionally, when a tube test for social dominance was conducted, *Fmr1* KO mice retreated more frequently than WT mice, indicating a possible increase in social anxiety.[22] However, despite the extensive studies conducted on adolescent humans with FXS, studies using juvenile mouse model of FXS are limited. Instead, there is a notable focus on adulthood. Particularly, social behaviors or the

age-related progression of behavioral changes have not been well investigated in *Fmr1* KO female mice, as most studies have focused on adult male mice.[23] Investigating early post-natal differences is crucial, since it allows for a deeper understanding of the relationship between the development of social and cognitive symptoms in FXS with age. It further allows for early identification for promising therapeutic interventions. FXS also represents a major genetic risk factor for ASD, in which this knowledge could help facilitate the implementation of early therapeutic interventions for children with ASD.[24] In this study, we aim to bridge the gap by focusing on social behaviors in both females and males of a mouse model of FXS at the juvenile stage.

The primary objective of this study was to conduct an in-depth analysis of the effect of genotype and sex on social behavior parameters in juvenile *Fmr1* KO mouse model of FXS, compared with the wildtype (WT) control. To achieve this goal, we utilized a free-form social interaction test, and quantified interactions over time across multiple key facets of social behavior using a recently developed marker-less tracking algorithm.[25] By investigating both female and male mice at a juvenile stage, we hope to uncover unique patterns of social behavior in FXS, and consequently shedding light on the complex mechanisms of this condition.

Methods

Animals:

Breeder wildtype (C57BL/6J, Jax stock No: 000664) and *Fmr1* knockout (B6.129P2-*Fmr1*^{tm1Cgr}/J, Jax stock No: 003025) mice were obtained from the Jackson Laboratory (ME, USA) and maintained at the mouse facility of the Cumming School of Medicine, University of Calgary. Behavioral testing was performed on male and female *Fmr1* KO and WT mice, bred in our animal facility at the University of Calgary. All KO mice of the current study were generated by mating *Fmr1* KO *(Fmr1 -/-)* homozygous females and *Fmr1* KO *(Fmr1 -/y)* males. Mice were group housed (up to five per cage) with their same-sex littermates. Cages were in a room with a controlled 12-hr light-dark cycle (lights on at 7:00am) with access to food and water ad libitum. Mouse pups were weaned around 20 days of age. All mice were fed standard mouse chow (standard diet). Behavioral testing was performed between 09:00h and 19:00h. All behavioral testing procedures were performed in accordance with the guidelines in the Canadian Council for Animal Care and was approved by the Health Sciences Animal Care Committee of the University of Calgary.

Social Interaction Behavioral Test:

For this experiment, 9 pairs of male WT mice, 11 pairs of male *Fmr1* KO mice, 7 pairs of female WT mice, and 6 pairs of female *Fmr1* KO mice were tested. At the start of testing, the mice were approximately 40 days old.

Social interaction was assessed in a 30 x 30 x 30 cm chamber, with an overhead camera attached to the lid. To conduct the social interaction test, two mice of the same age, sex, and genotype but from different cages, were both placed into the testing cage. Before the test, neither male nor female mice had been exposed to the opposite sex after weaning. The mice were group-housed and habituated to the behavioral testing room in their respective home cages for 30 minutes before testing. The matched mice were then transferred to the

social tracking chamber for testing. Testing sessions were recorded for 10 minutes with Spinview software. The chamber was cleaned with 70% ethanol and left to dry for several minutes before the start of subsequent tests.

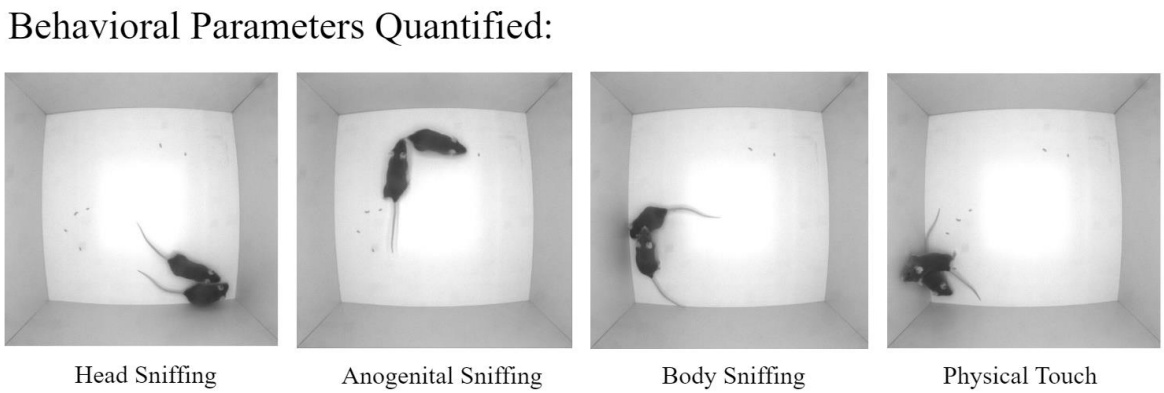

Figure 1. Real time images of the behavioral parameters. These four real time images illustrate the parameters quantified, specifically, for head sniffing, anogenital sniffing, body sniffing, and physical touch.

Behavioral Analysis:

The coordinates of each mouse's snout and tail-base, as well as the body mask, were generated using the marker-less tracking algorithm.[25] These parameters were then used to determine social investigation of each mouse towards the other. Specifically, the amount of time the snout of each mouse was directed towards the ano-genital, body, or head/face region of the other mouse was quantified. If one mouse's snout was within a 25-pixel threshold distance from the other mouse's snout, tail-base, or body, a behavioral label was given. Since our approach generates body masks in addition to the snout and tail-base coordinates, we could also determine how much time the pair of mice spent in contact with each other by detecting at what times the masks overlapped. By removing the snout-directed social investigation times from this data, we could determine the amount of time each pair spent touching that was not because of snout-directed investigation. Thus, we analyzed four types of social behaviors including: head sniffing, anogenital sniffing, sniffing of any part of the partner's body excluding head and anogenital area (body sniffing), and physical touch. For each behavior, we quantified the total time each animal spent in doing that behavior, the occurrence count of that behavior, the average duration of each occurrence, and the average interval between occurrences. We also used the tracking algorithm to measure the speed as well as distance and trajectory travelled by each animal.

Statistical Analysis:

Statistical tests were performed in GraphPad Prism 10.1.0 (GraphPad Software, San Diego, California), and Figures were generated in GraphPad Prism and Excel Spreadsheet. All statistical analyses are presented in the Figure legends and Tables. All data is shown as Mean ± SEM and α was set at 0.05. A two-way ANOVA test was performed, comparing the variables "genotype" and "sex." Subsequently, a post-hoc analysis using the Tukey method was conducted.

Results

For head sniffing behavior, there were significant sex difference for all parameters measured, and a genotype difference between the female KO and female WT mice in interval between single event and number of total events.

We first quantified head sniffing behavior (**Fig. 2**). The total time spent in this behavior was shorter in females compared to males **(A)**. Female WT had a significantly shorter duration compared to male WT, however no differences were found when comparing female KO with male KO **(A)**. Further, there were no genotype differences present between the female WT and female KO, as well as between the male WT and male KO **(A)**. The accumulative time spent in head sniffing with their partners followed a gradual increase over time for both sexes and genotypes **(B)**. Notably, females showed a greater time spent in a single event compared to males **(C)**. Specifically, female WT had a longer duration of a single head sniffing event compared to male WT, and female KO showed greater time compared to male KO **(C)**. In addition, there is a significant genotype difference present between the female WT and female KO: on average WT mice spent more time in a single event than KO mice **(C)**. The interval between single events was longer in females than males **(D)**. Specifically, it was longer in female WT mice than male WT mice **(D)**. Further, female WT had a significantly longer interval between single events compared to female KO **(D)**. Finally, females displayed a lower number of total head sniffing events compared to males, with female WT having a smaller count compared to male WT mice **(E)**. In addition, female WT exhibited a lower count compared to female KO mice **(E)**.

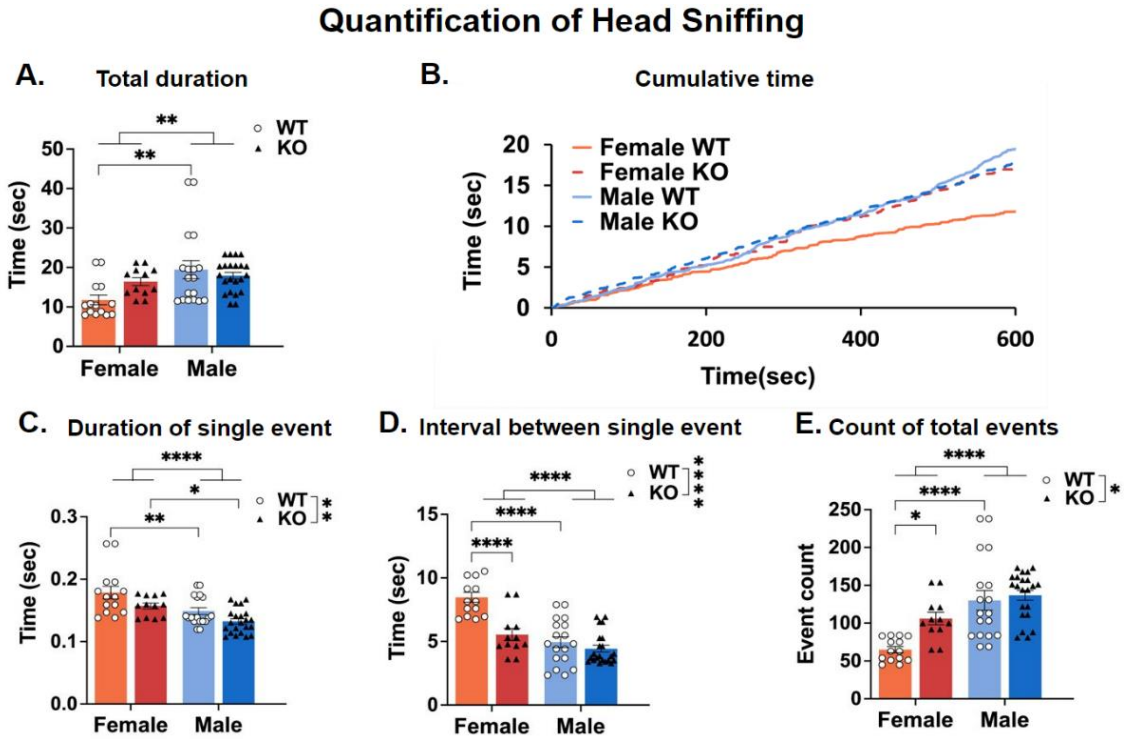

Figure 2. Quantification of head sniffing behavior parameters of juvenile mice in 10-minute recordings. This figure shows the total duration of social interactions (A), cumulative time (B), duration of single event (C), interval between single events (D), and the

count of total events in juvenile WT (circles) and *Fmr1* KO (triangles) (E). Juvenile females, WT (n=14) and KO (n=12); juvenile males, WT (n=18) and KO (n=22). Data is expressed as mean (bars) ± SEM (error bars) and individual animals (symbols). p value was $p < 0.05$.

For anogenital sniffing behavior, there were significant sex differences in all the parameters measured and a genotype difference between the female WT mice and female KO mice.

We then quantified anogenital sniffing behavior (**Fig. 3**). The total duration spent in this behavior with its partner was shorter in females compared to males, particularly with female WT mice showing shorter duration compared to male WT mice **(A)**. In addition, female WT mice had shorter total duration than the female KO mice **(A)**. There were no significant differences observed between male WT and male KO **(A)**. The accumulative time spent in anogenital sniffing with their partners followed a gradual increase over time for both sexes and genotypes **(B)**. Interestingly, the female mice had a longer duration of a single anogenital sniffing event compared to the males **(C)**. Additionally, female mice showed longer interval between single events compared to their respective male counterparts **(D)**. The female WT mice had greater interval compared to male WT, and further, female KO had greater interval compared to male KO **(D)**. Moreover, genotype differences were present when measuring the interval between single anogenital sniffing events: female WT had longer interval compared to female KO **(D)**. Finally, females showed a lower number of total events compared to males, with female WT having a smaller count compared to male WT mice, and female KO having a smaller count than male KO mice **(E)**. Further, female KO mice exhibited a larger count compared to female WT mice **(E)**.

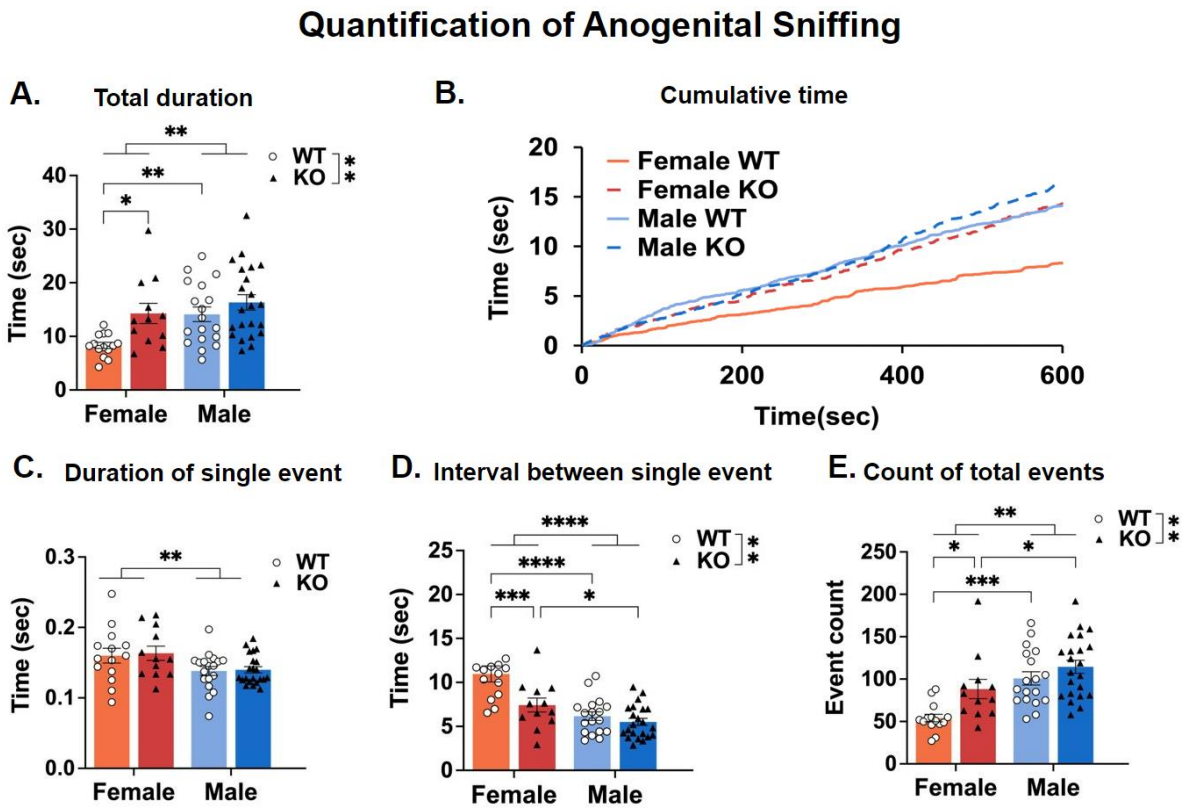

Figure 3. Quantification of anogenital sniffing behavior parameters of juvenile mice in 10-minute recordings. This figure shows the total duration of social interactions, cumulative time, duration of single event, interval between single events, and the count of total events in juvenile WT (circles) and *Fmr1* KO (triangles). Juvenile females, WT (n=14) and KO (n=12); juvenile males, WT (n=18) and KO (n=22). Data is expressed as mean (bars) ± SEM (error bars) and individual animals (symbols). *p* value was $p < 0.05$.

When quantifying body sniffing behavior, we found a sex difference in each of the parameters except duration of single interaction event, and further genotype differences between female KO and WT mice in total duration and count of body sniffing behavior.

We quantified body sniffing behavior (**Fig. 4**). The total duration spent in this behavior with its partner was shorter in females compared to males, particularly with female WT mice showing shorter duration compared to male WT mice **(A)**. Further, there were genotype differences present between the female WT and female KO: the WT showed less body sniffing activity compared to KO **(A)**. The accumulative time spent in body sniffing with their partners followed a gradual increase over time for both sexes and genotypes **(B)**. No significant differences were observed between the experimental groups **(C)**. The female mice showed longer intervals between single body sniffing events compared to their respective male counterparts **(D)**. Specifically, the female WT mice showed a longer time interval spent between single events compared to male WT mice **(D)**. Additionally, the female WT also showed greater interval between events compared to the female KO mice **(D)**. When

considering the number of total events, females showed a smaller number of total events compared to males, with female WT having a lower count than male WT mice **(E)**. Moreover, female WT mice exhibited a significantly smaller count compared to female KO mice **(E)**.

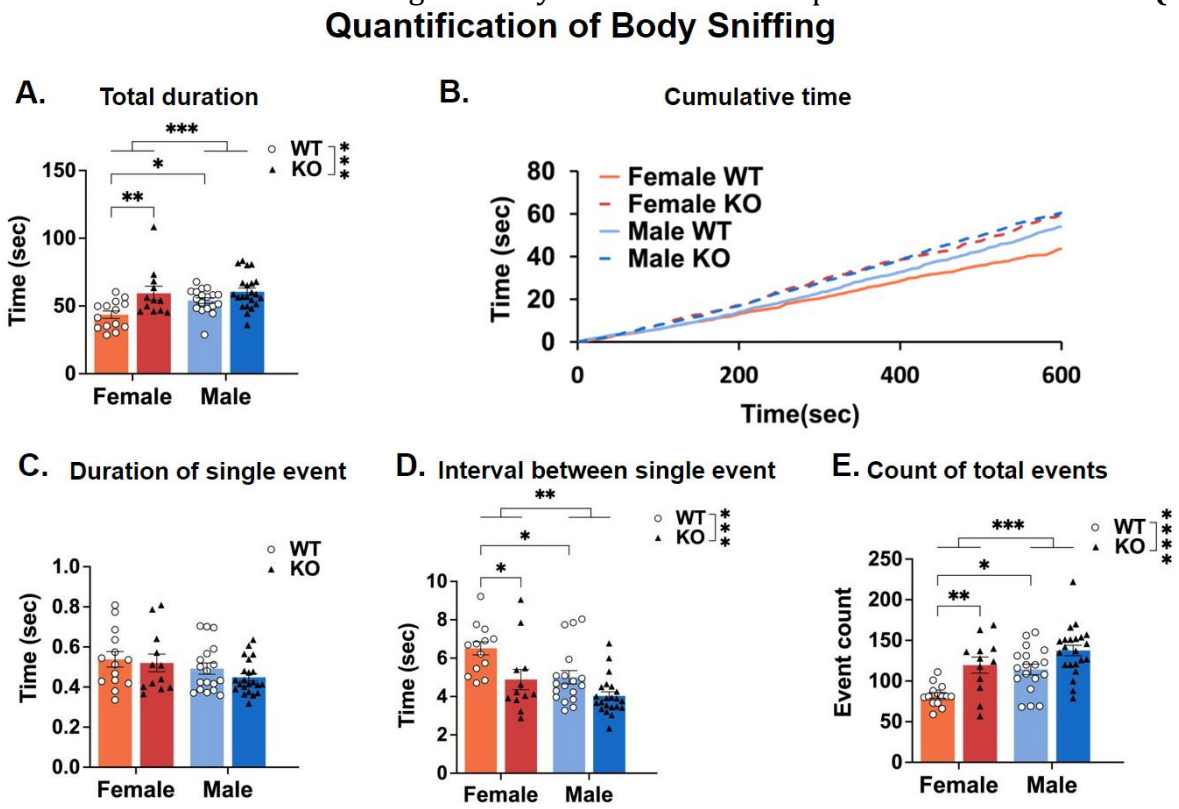

Figure 4. Quantification of body sniffing behavior parameters of juvenile mice in 10-minute recordings. This figure shows the total duration of social interactions, cumulative time, interval of single event, interval between single events, and the count of total events in juvenile WT (circles) and *Fmr1* KO (triangles). Juvenile females, WT (n=14) and KO (n=12); juvenile males, WT (n=18) and KO (n=22). Data is expressed as mean (bars) ± SEM (error bars) and individual animals (symbols). *p* value was $p < 0.05$.

During physical touch behavior, there was a sex difference for each of the parameters, as well as a genotype difference between the female KO and female WT except in duration of a single interaction event.

We quantified physical touch behavior (**Fig. 5**). The total duration spent in this behavior with its partner was shorter in females compared to males, particularly with female WT mice showing shorter duration compared to male WT mice **(A)**. Further, there is a genotype difference present in the female mice: the female WT mice showed less total interaction than female KO mice **(A)**. The accumulative time spent in physical touch with their partners followed a gradual increase over time for both sexes and genotypes **(B)**. Females showed slightly longer duration of single physical touch event compared to males **(C)**. Further, female WT showed significantly more time in a single event compared to female KO **(C).** The female mice showed longer interval between single events compared to their respective male counterparts **(D)**. The female WT mice showed a longer time interval spent between single events than male WT mice **(D)**. Further, female WT mice showed significantly more

time between single events compared to female KO mice **(D)**. When considering the count of total events, females showed a lower number of total events compared to males, with female WT having a lower count compared to male WT mice **(E)**. In females, WT mice showed a smaller number of total events than female KO **(E)**.

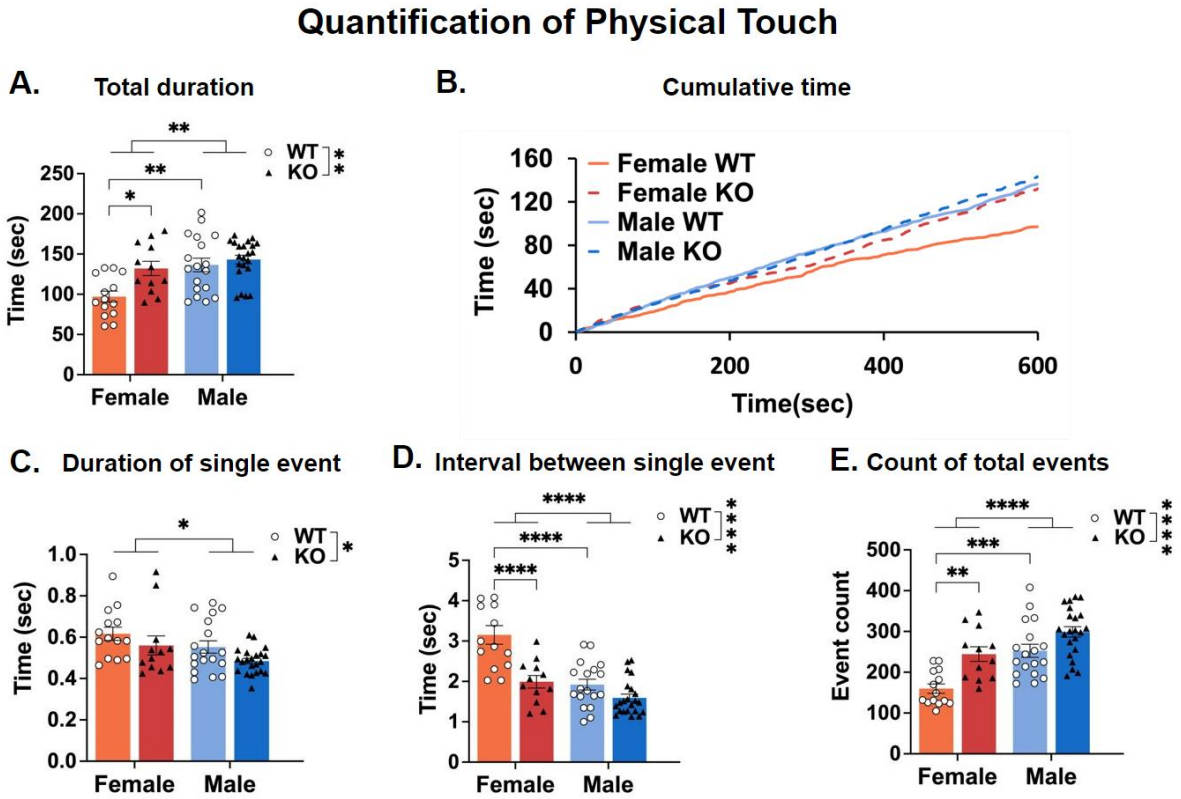

Figure 5. Quantification of physical touch behavior parameters of juvenile mice in 10-minute recordings. This figure shows the total duration of social interactions, cumulative time, duration of single event, interval between single events, and the count of total events in juvenile WT (circles) and *Fmr1* KO (triangles). Juvenile females, WT (n=14) and KO (n=12); juvenile males, WT (n=18) and KO (n=22). Data is expressed as mean (bars) ± SEM (error bars) and individual animals (symbols). *p* value was $p < 0.05$

When considering the distance travelled by the mice, there was a sex difference when quantifying the total distance travelled, where males showed, a higher distance travelled compared to females, with significant differences between male KO and female WT.

The distance travelled was then quantified (**Fig. 6**). Both sex and genotype differences were present when observing the total distance travelled **(A)**. The accumulative distance travelled followed a gradual increase over time for both sexes and genotypes **(B)**. Tracking trajectories for each group were quantified by the algorithm software, with blue lines representing the body center of one mouse and red tracing the other mouse **(C-F)**.

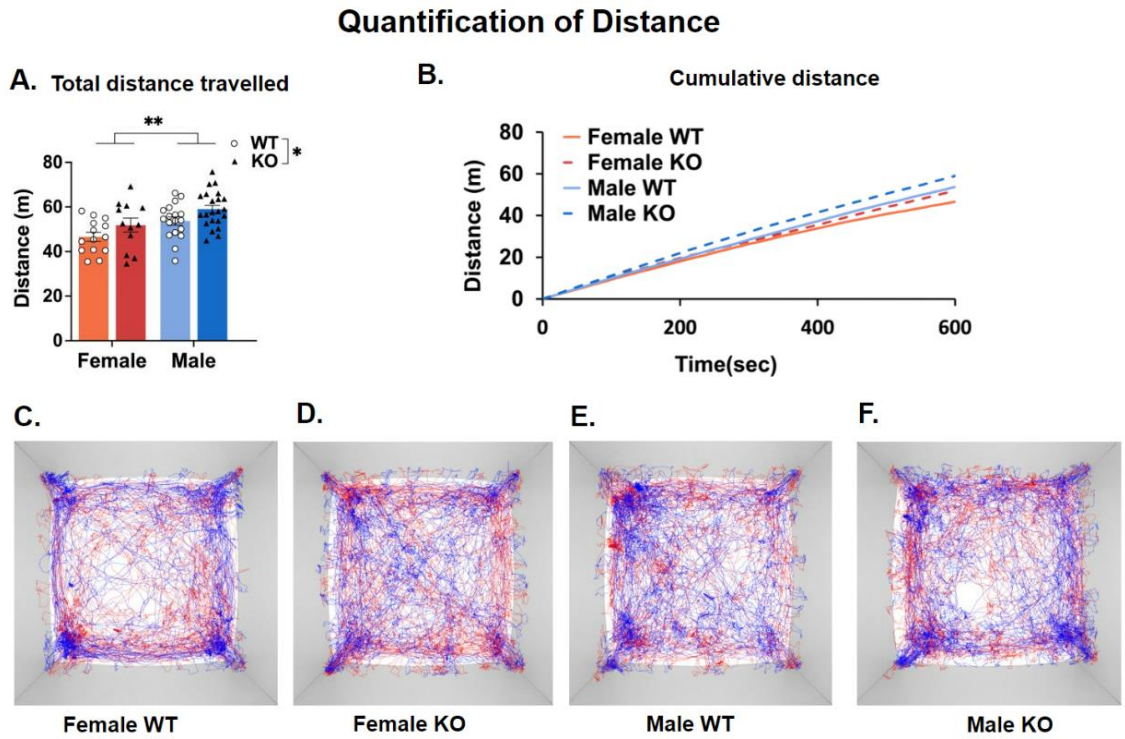

Figure 6. Quantification of distance travelled of juvenile mice in 10-minute recordings. This figure illustrates the total distance travelled, cumulative distance over time, and tracking trajectories in juvenile WT (circles) and *Fmr1* KO (triangles). Juvenile females, WT (n=14) and KO (n=12); juvenile males, WT (n=18) and KO (n=22). Data is expressed as mean (bars) ± SEM (error bars) and individual animals (symbols). *p* value was $p < 0.05$

Overall, sex differences are found in all the behavioral parameters, specifically in the total duration of social interaction. Additionally, each parameter contained significant differences between the WT and KO females by post hoc pairwise comparisons, specifically in total duration of interactions, interval between events, and interval count number. On the other hand, there is no significant differences observed between the WT and KO males. Further, all experimental groups showed a gradual increase in the time spent socially interacting, as well as distance travelled, over time.

Table 1. Data summary of behavioral parameters at the juvenile stage

		Sex		**Genotype**	
		WT females vs. WT males	KO females vs. KO males	WT females vs. KO females	WT males vs. KO males
Total duration	Head sniffing	**			
	Anogenital sniffing	**		*	

	Body sniffing	*		**	
	Physical Touch	**		*	
Duration of single event	Head sniffing	**	*	**	
	Anogenital sniffing				
	Body sniffing				
	Physical Touch				
Interval between single events	Head sniffing	****		****	
	Anogenital sniffing	****	*	***	
	Body sniffing	*		*	
	Physical Touch	***		***	
Count of total events	Head sniffing	****		*	
	Anogenital sniffing	***	*	*	
	Body sniffing	*		**	
	Physical Touch	***		**	

Discussion

Social interactions are a foundational anchor in society and serve as the building block to create a plethora of stable networks and relationships with others. It encompasses multiple individuals exhibiting a diverse and complex range of behaviors affected by their individual emotional states and motivations.[7] Differences in social behaviors, especially social anxiety and withdrawal, are prominent features of FXS. In addition, the underlying mechanisms behind the differences in social behavior observed in FXS patients remains unclear, hence there are no effective interventions that can help patients to overcome social challenges. As a result, having a precise, comprehensive characterization of social behavior is necessary to understand the neurobiological basis that underlies its complexities.

Several studies have shown that *Fmr1* KO mice can be used to replicate some of the behavioral defects of autism and FXS.[26,27] Therefore in this study we compared the social behaviors of both male and female mice of the *Fmr1* KO mouse model of FXS with WT mice through the analysis of several behavioral parameters at the juvenile stage. Juvenile mice provide a unique opportunity to improve our understanding of the importance of early

intervention and mitigation of long-term impacts, since this age is considered a sensitive period of the brain development and behavioral plasticity in both mice as well as in humans.[28] Moreover, studies in humans have shown that young children with FXS may provide a unique understanding of the early signs of developing multiple psychiatric disorders, such as ASD, social anxiety, and generalized anxiety disorder as they grow older.[11]

Sociability studies like the three-chambered sociability task were used in previous studies, to study the behavior of a subject mice when allowed to interact with a novel mouse against a novel object.[29] In general, previous studies that used the three-chambered sociability tests to study the behavior of *Fmr1* KO mice observed varying social behaviors.[21] Some studies observed that *Fmr1* KO mice were similar to their wild-type counterparts and preferred to interact with the novel mouse rather than the novel object.[21,27,30,31] Other studies observed that *Fmr1* KO mice displayed increased social interaction.[22] One study noted enhanced locomotor activity and increased social interest in the three-chambered sociability task in *Fmr1* KO mice which may be explained by the mice's more frequent interaction with the stranger mouse relative to the WT mice.[32] On the other hand, other studies concluded that *Fmr1* KO mice may not have any preference for the novel mouse over the novel object, which is also seen with decreased duration of sniffing events of the novel mouse in the three chambered sociability tests.[30] Another study mentions that it is unlikely that hyperactivity reflects the differences in results during the 3-chamber test because there were no differences in crossing the arenas during the habituation and testing phases.[34] Rather, a more likely explanation is that the differences in social interaction are because of greater arousal/anxiety in the *Fmr1* KO mice than for the WT, making it difficult for them to stay in contact with the stimulus mouse.[34]

In mice, socio-emotional interactions are mediated by various modalities such as visual, olfactory, and auditory senses.[7] Sniffing is a key active behavior to acquire odors and pheromones exhibited during social behaviors.[34] The mechanism by which mice communicate is not fully understood, however, exploration of a novel context through sniffing is a measurement for the arousal state of the animal and is relevant to information exchange between brain areas.[7] In mice, sniffing behavior and close physical contact appear to contribute to communication of meaningful information and social hierarchy.[35] Previous studies that analyzed reciprocal social interactions like nose-to-nose sniffing, nose-to-head sniffing and body sniffing of *Fmr1* knockout mice models generally linked a reduction in sniffing time with sociability deficits.[36,37] Although some sniffing behavior like anogenital sniffing has been correlated with the collection of odor information and sexual interest, the reason underlying other individual sniffing behaviors remains unexplored.[38]

Deep learning approaches, as displayed by the software used in the present study (REF), enabled the detection and tracking of changes in the positions of defined body parts, that allowed for the investigation of social interactions in a more natural setting.[39] In this study utilizing the FXS mouse model, the length of social interaction following initiation of an approach depends on the willingness of both mice, as discrepancies in the cooperation or interest of a mouse will result in a shorter interaction. Furthermore, the number of social interaction events a mouse engages in correlates to their interest levels: more counts of events may correlate to increased social interest. Our results on male and female *Fmr1* KO

and WT mice on a C57BL/6J background were complex, in terms of the level of social interactions in a 1-to-1 setting. Based on the analysis of all four behaviors (head sniffing, anogenital sniffing, body sniffing, and physical touch), there were robust sex differences: females exhibited a longer duration in a single interaction event as well as longer intervals between events, for all the parameters except for body sniffing; in contrast, the male mice had significantly more interaction counts than the female cohorts. In terms of genotype differences, in all the social behaviors we quantified, the KO mice displayed significantly more interaction events than the WT mice, especially comparing female KO and WT mice. In a study by McNaughton et al., where adult male mice of age 8-10 months were tested in the three-chambered apparatus, *Fmr1* KO mice had shorter average duration of nose contact interactions and higher levels of grooming during initial interactions.[33] These findings are consistent with the findings in the present study, where KO mice displayed a shorter duration of a single event during head sniffing and physical touch, compared to the WT counterparts, and may reflect a common feature in this mouse model of FXS. Furthermore, our results showed that the in terms of sex differences, female WT mice had a smaller count of social interaction events than their male WT counterpart in all the behaviors measured. There were also genotype differences between the female WT and female KO for the count of total events in the social interaction test. On the other hand, there were no significant differences between the male KO and WT mice, and this may be due to potential sex differences, not analyzed in this experiment, such as stress-enhanced affiliative behaviors.[40]

Our findings also suggest that locomotor hyperactivity of the FXS mice may contribute to but is unlikely to completely account for the increased social interaction events observed in this study. Our results indicated that there were both sex and genotype differences in the total distance travelled, wherein male mice travelled a larger distance compared to females and the *Fmr1* KO genotype exhibited increased movement compared to their WT counterparts. However, post hoc pairwise comparison did not reveal any significant difference between female WT and female KO mice in distance travelled. Yet, female mice displayed significant genotype difference in the four social behavior parameters. Thus, the increased social interaction events of the female KO mice likely involve other factors such as changes in anxiety levels, altered social motivations, or other mechanisms which may not be directly linked to locomotor activity.

The results in our study can be extended to assess its applicability in studies involving children with FXS. Cregenzan-Royo et al. reported that about 21.5% and 17.5% of boys and girls with FXS, respectively, between 6 and 17 years of age displayed social withdrawal, while another recent study by Hessl et al. showed that 40% of girls and 41.8% of boys (aged 6-16 years) with FXS had social problems in the clinical range.[1,41] Poor socialization skills have been associated with adverse outcomes, including symptoms of anxiety and/or depression, reduced adaptive skills, and decreased quality of life.[18] In studies of FXS patients, Hong et al. used emotional face gaze analysis in patients between 5-30 years of age to study social interest and anxiety symptomology and found that both occur and contribute to social difficulties in individuals with FXS.[42] The study found that social impairment in FXS patients were more related to anxiety than social disinterest, as shown by gaze aversion and social avoidance.[42] Interestingly, it also found that greater social preference was correlated with more severe anxiety in FXS patients.[42] This may be due to socially anxious individuals being

more hypervigilant to surrounding stimuli or because individuals with FXS that have a higher level of social interest are more susceptible to social anxiety compared to their less socially cognizant peers.[42] These findings are overall consistent with studies reporting that individuals with FXS show behaviors suggestive of a willingness and motivation to interact with others, despite their social communication impairments and anxiety. [43]

Limitations and Future Directions:

A challenge in behavioral analysis pertains to group dynamics. This study focused on interactions between two mice, so the behavior of mice in this dyadic circumstance is not accurately predictable. This is because certain aspects of social behavior are influenced by interactions that do not just involve another individual. The bulk of research on animal models relies on behavioral paradigms, thus hindering our understanding of the neural mechanisms governing other aspects of social behavior in more complex settings. For example, emotions greatly influence behavior, yet most studies, including this one, do not include a reliable means for assessing the emotional state of the mice undergoing the social interaction test. Further, the social behavior of a tested mouse may be affected by variables including established dominance, social rank and individual temperaments.[44] Thus, performance in these tests may be influenced by multiple variables.

Other future research includes further analysis on brain activity in the mice during social interactions. This can be done through recording neural activities from brain regions involved in social interaction, such as prefrontal cortex and amygdala, which may give clues to whether the mice have more motivation and/or anxiety. Additionally, several researchers have suggested that social avoidance behavior in FXS may result from a dysregulation of the sympathetic and parasympathetic nervous systems, providing a new explanation for why people with FXS often seem overly anxious or agitated during social interactions.[45] Another study by Belser and Sudhalter reported that tonic skin conductance, which reflects activity of sweat glands, of male subjects with FXS were significantly elevated during direct eye contact with others, indicating potential maladaptive responses to social cues.[46] Additionally, a study by Miller et al. found that electrodermal responses to sensory stimuli tests, also indicating sweat gland activity and further sympathetic system activity, were elevated.[47] Further, inversely proportional ratios to the levels of FMRP were detected, such that people with higher levels of FMRP were associated with a more typical electrodermal response.[47] Several studies have observed heightened physiological arousal in FXS patients during cognitively demanding tasks.[48,49] These observations include significantly elevated heart rates, indicative of sympathetic nervous system activity, and significantly lower vagal tone, a measure of parasympathetic nervous system activity.[48,49] These differences are consistently seen in individuals with FXS when compared to a group of age-matched typically developing children, both at baseline and during completion of cognitive tasks.[48,49] Based on these studies, it is likely that investigating arousal states of animal models can provide new understanding of social behaviors in both typical and altered states.

Conclusions

This study demonstrated the differences associated with various social behaviors in the juvenile *Fmr1* KO mouse model of FXS. Together, our data provides evidence for the

presence of behavioral discrepancies in female mice, highlighting the importance of considering age and sex differences when using this mouse model. These results also suggest that investigating the early post-natal phases is crucial for potential preventative interventions. Future studies are necessary to further reveal the cognitive and neural processes governing normal social behaviors and can aid in understanding how these mechanisms may produce differences in social behavior.

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