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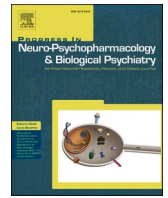


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Adaptive group behavior of Fragile X mice in unfamiliar environments

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ABSTRACT

Fragile X Syndrome (FXS) stands out as a prominent cause of inherited intellectual disability and a prevalent disorder closely linked to autism. FXS is characterized by substantial alterations in social behavior, encompassing social withdrawal, avoidance of eye contact, heightened social anxiety, increased arousal levels, language deficits, and challenges in regulating emotions. Conventional behavioral assessments primarily focus on short-term interactions within controlled settings. In this study, we conducted a comprehensive examination of the adaptive group behavior of *Fmr1* KO male mice over a three-day period, without introducing experimental interventions or task-based evaluations. The data unveiled intricate behavioral anomalies, with the most significant changes manifesting during the initial adaptation to unfamiliar environments. Notably, certain behaviors exhibited a gradual return to typical patterns over time. This dynamic *Fmr1* KO phenotype exhibited heightened activity, featuring increased exploration, amplified social interest, and an unconventional approach to social interactions characterized by a higher frequency of shorter engagements. These findings contribute to the growing understanding of social behavior in individuals with FXS and underscore the significance of comprehending their adaptive responses in various environmental contexts.

1. Introduction

Fragile X Syndrome (FXS) stands as the primary cause of inherited intellectual disability (ID) and ranks as the most prevalent syndrome related to autism spectrum disorders (ASD) (Hunter et al., 2014; Wiśniowiecka-Kowalnik and Nowakowska, 2019). It affects approximately 1.4 in 10,000 males and 0.9 in 10,000 females (Hunter et al., 2014). FXS is the result of a mutation in the *FMR1* gene, which impedes the production of Fragile X Messenger Ribonucleoprotein 1 (FMRP), a crucial protein for neurodevelopment and the maintenance of neuronal and synaptic functions (Richter and Zhao, 2021). Insufficiency of FMRP leads to significant abnormalities in the central nervous system, contributing to the cognitive and behavioral disorders observed in individuals with FXS (Hagerman et al., 2017). The FXS phenotype is characterized by significant alterations in social behavior, which profoundly impact the well-being of individuals and their families. These

symptoms encompass social withdrawal (Kau et al., 2004; Kaufmann et al., 2004; Budimirovic et al., 2006), avoidance of gaze (Cohen et al., 1988; Farzin et al., 2011; Hall et al., 2015), social anxiety (Crawford, 2023; Cordeiro et al., 2011; Bailey et al., 2008), hyperarousal (Cohen, 1995), deficits in language development (Abbeduto et al., 2007; Finestack et al., 2009), and difficulties in recognizing and regulating emotions (Hagan et al., 2008; Shaw and Porter, 2013). These symptoms collectively contribute to the deterioration of the social competence of individuals with FXS.

Research on FXS in mice has primarily relied on the initial *Fmr1* knockout model (*Fmr1* tm1Cgr) (The Dutch-Belgian Fragile X Consortium et al., 1994), characterized by insertional inactivation of exon 5 in the *Fmr1* gene and resulting in the loss of FMRP. However, this model retains an active *Fmr1* promoter, leading to the production of abnormal residual *Fmr1* RNA. To address this, a new *Fmr1* mouse mutant was created (Mientjes et al., 2006), known as *Fmr1* KO2, which

Abbreviations: FXS, Fragile X Syndrome; ID, intellectual disability; ASD, autism spectrum disorders; *Fmr1*, Fragile X Messenger Ribonucleoprotein 1 gene; FMRP, Fragile X Messenger Ribonucleoprotein 1; WT, wild type; KO, knockout; LMT, Live Mouse Tracker; PCA, Principal Component Analysis; S-S, side-by-side; N-AG, nose-to-anogenital; N-N, nose-to-nose.

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completely abolishes the transcription of *Fmr1* mRNA. The latter model was employed in this study and will hereafter be referred to as *Fmr1* KO.

Assessing mouse social behavior under both normal and pathological conditions is crucial for gaining insights into the neural systems implicated in psychiatric disorders. Many studies have used standardized short-term social interaction assessments to investigate the social interaction behavior of FXS mouse models (Sørensen et al., 2015; Liu and Smith, 2009; Hébert et al., 2014; Heitzer et al., 2013; McNaughton et al., 2008; Yang et al., 2022; Gaudissard et al., 2017). The studies have reported various alterations in the social behavior of these mice, but the results have shown heterogeneity, likely attributable to differences in the protocols used (Rein et al., 2020). Standardized tests primarily assess parameters related to simple, short-lived social interactions occurring in controlled, unfamiliar settings. Additionally, within the context of ASD, the experimenter manipulation and the unfamiliar settings typically encountered in task-based assessments can act as significant stressors, profoundly impacting animal behavior and hindering the observation of complex, naturalistic behaviors (Sorge et al., 2014; Jabarin et al., 2022; Kondrakiewicz et al., 2019). Furthermore, this approach lacks important ethological behavioral markers, such as group dynamics and long-term interactions (Olsson et al., 2003).

In this study, we leveraged the Live Mouse Tracker (de Chaumont et al., 2019), a tool that facilitates continuous, unsupervised, and non-invasive monitoring of spontaneous social interactions among groups of wild-type and *Fmr1* KO mice. Our objective was to explore the social behavioral traits associated with FXS within an environment designed to mimic their standard housing and enrichment conditions. This innovative approach removes the necessity for task-based assessments and allows for the analysis of group behaviors over extended time periods.

The data presented here unveils a spectrum of intricate anomalies within the exploratory and social behavior of *Fmr1* KO mice. The most pronounced variations were observed during their initial adaptation to the unfamiliar environment. However, some altered behaviors exhibited a gradual attenuation, eventually normalizing within a two-day period. This dynamic FXS phenotype manifests as a hyperactive profile, marked by a heightened propensity for exploring new surroundings, an increased interest in social interaction combined to a distinctive pattern of social engagements characterized by more frequent but shorter interactions in comparison to the control group.

2. Methods

2.1. Animals

Animals were treated in compliance with the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health Guide for the care and use of laboratory animals. The French Ethical committee authorized this project (APAFIS#34573-202201071122121v3). *Fmr1* KO2 mice from FRAXA foundation were used in this study. Females *Fmr1*+/- were paired with males *Fmr1*+/*y*, both with C57Bl/6 J background. Pups were weaned and ear punched for identification and genotyping at postnatal day 21 (P21). *Fmr1*+/*y* mice composed the control group (wild type (WT)) and *Fmr1*-/*y* the experimental group (knockout, KO). Mice were housed in standard wire-topped Plexiglas cages (42 × 27 × 14 cm) in a temperature and humidity-controlled condition (i.e., temperature 21 ± 1°C, 60 ± 10% relative humidity and 12 h light/dark cycles). Food and water were available *ad libitum*.

2.2. Live mouse tracker

To perform real-time behavior acquisition/analysis of group-housed mice the Live Mouse Tracker (LMT), leverages infrared tracking, RFID-chip identification, and machine learning (de Chaumont et al., 2019). The LMT arena is a 50x50x30cm cage made in transparent PMMA. For each LMT run, the arena was covered with 1 kg of litter. Two squared

houses (10x10x5cm) of red transparent PMMA were placed in two opposite corners of the arena. Two cotton rolls were placed next to each house. A U-shaped house (7.62 × 9.5 × 4.5 cm) of red transparent PMMA was placed between the two squared houses for enrichment. 100 g of standard chow (an *ad libitum* quantity for the experiment duration) was placed in the arena. Mice had *ad libitum* access to two bottles of drinking water placed in the two corners opposite to squared houses. The disposition of the elements in the arena is shown in Fig. 1A. The center zone was defined as a 30x30cm area.

Forty mice (20 WT and 20 KO) aged between P70 and P90 were used in this study, divided into 10 experimental groups (5 WT and 5 KO) of 4 mice each. Each group consisted of 2 pairs of littermates from two litters born within 3 days of each other, which were housed together in the same cage from weaning until the day of the experiment.

The activity of *Fmr1* WT or KO mice was studied in homogeneous groups of four mice of the same sex and genotype in a custom-built arena (Fig. 1A–C) that mimicked their housing conditions. The experiment consisted of a continuous 64-h recording period, starting with a 4-h acclimation phase to the new environment. Subsequently, the recording period was divided into three 12-h nocturnal (i.e. active) phases alternated with two 12-h diurnal (i.e., inactive) phases (Fig. 1D). Throughout the experiment, each mouse was continuously monitored along the x, y, and z axes, allowing for the construction of a comprehensive behavioral profile based on the detection of various exploratory and social parameters (Fig. 1E).

Our strategy considered the natural tendency of male mice to compete for territory, resources, and dominance rank. Specific measures taken to mitigate aggression included: -Strain Selection: We used the C57Bl/6 J strain, known for being among the least aggressive mouse strains. -Resource Availability: Food and water were provided *ad libitum* and distributed across multiple zones of the arena, ensuring ample resources. -Environmental Enrichment: The environment was enriched with three different hiding devices and abundant nesting material. -Social Housing: Male mice were co-housed in groups of four before sexual maturity for several weeks prior to the LMT experiment. This approach aimed to establish stable social hierarchies, as studies have shown that smaller group sizes (5 or less) experience fewer agonistic incidents (Poole and Morgan, 1973). -Cage Cleaning Protocol: To eliminate olfactory familiarity and minimize aggression after cage cleaning, we implemented a complete and thorough cage change, ensuring no transfer of familiar scents. Moreover, manual scoring of aggressive events was performed within the first 30 min of the test, revealing no significant differences between genotypes (Supplementary Fig. 2). This period was chosen based on the fact that aggression in mice typically peaks around 15 min after a change in the environment (Gray and Hurst, 1995), making it a relevant window for observing such behavior (Van Loo et al., 2003). Aggressive episodes, or agonistic behavior, was assessed per group by counting instances where a mouse displayed specific behaviors toward conspecifics, including sideways threats, chasing, defensive upright postures, tail rattling, and attack bites.

Sound, humidity, temperature, and luminosity were monitored during experiments. No access to the LMT room was allowed during trial. Mice weight, food and water consumption were monitored before and after the experiment.

The recording protocol allowed us to capture three active periods. This duration is consistent with previous studies utilizing the LMT approach. Thus (de Chaumont et al., 2019), recorded for up to 3 days *Shank3* mice, while (Maisterrena et al., 2024) conducted experiments for 30 min within the light cycle and for 3 continuous days (72 h). Similarly (de Chaumont et al., 2021), and (Rusu et al., 2023) stopped recordings after 71 h.

2.3. RFID

Adolescent male mice (P30–35) were deeply anesthetized with

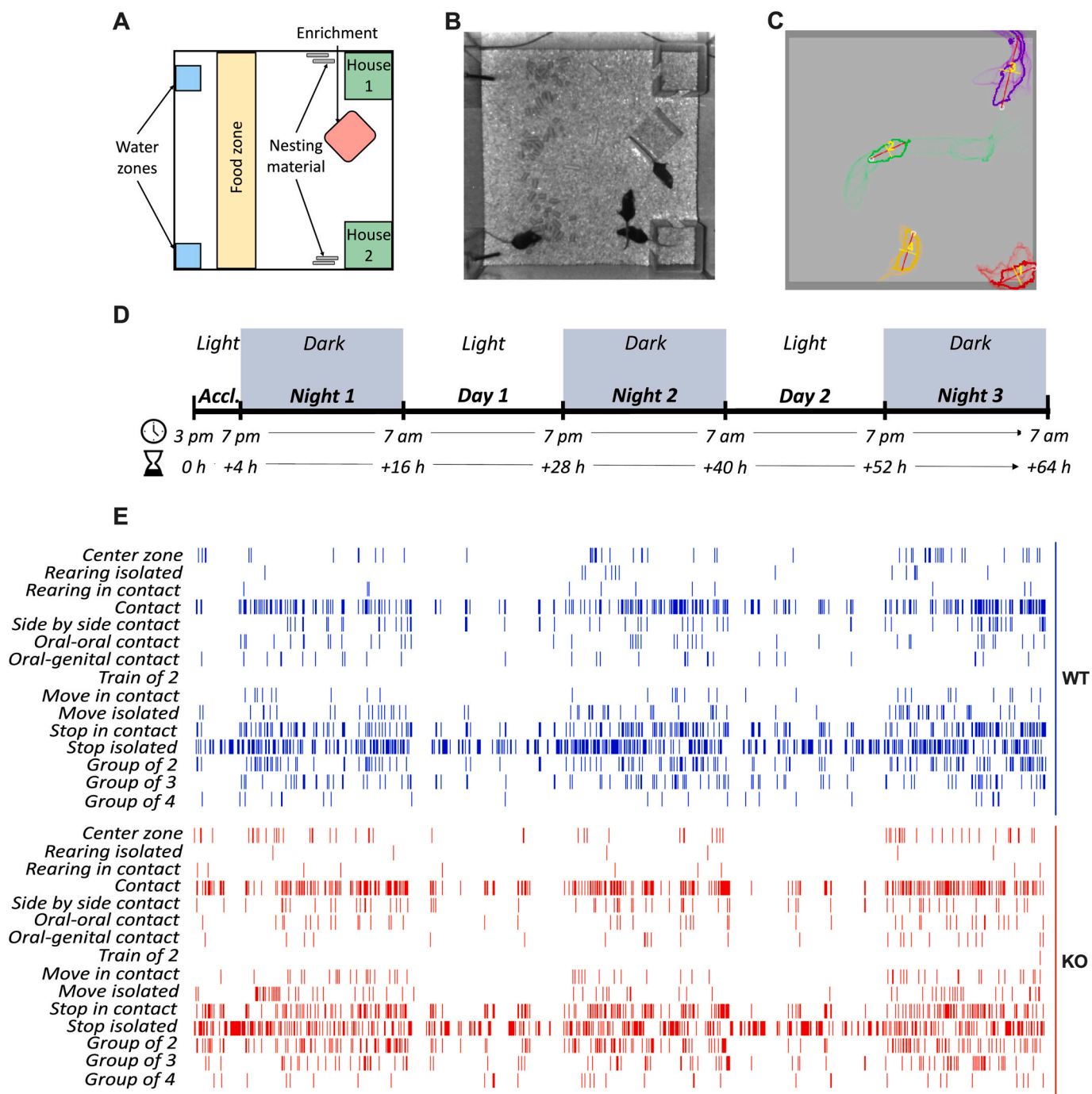


Fig. 1. Experimental setting. A. Scheme of the arena showing 2 accesses to 2 water bottles (blue), 1 area representing the food distribution area (yellow), 2 houses (green), environmental enrichment (red) and nesting material (gray). B. Picture of the arena and a group of 4 mice during the experiment. C. Live tracking of the mice, representing the mask and trajectory of each. D. Experimental timeline representing the light/dark cycles over the duration of the experiment. E. Example of the detection of different behaviors of a single mouse throughout the entire experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isoflurane and subcutaneously injected with a RFID chip (Biomark, APT12) using Biomark MK25 PIT Tag, behind the ears of the animal, and gently pushed to the back of the animal.

The RFID implantation was performed minimum 35 days before the LMT trial to avoid injection-induced stress impact on mouse behavior.

2.4. Data analysis

During each trial, the movements of each mouse within a social group of 4 individuals were tracked capturing 30 frames per second.

LMT data were extracted from the SQLite database using scripts from either the LMTAnalysisMaster packages or newly created scripts available on GitLab (https://gitlab.com/chavis_manzoni_lab/lmt-scripts), both utilized with Pydev in Eclipse. Several types of behaviors were extracted from the dataset (list and descriptions is provided in supplementary information in (de Chaumont et al., 2019)).

2.5. Statistics and graphs

Three periods of interest for statistical analysis were selected based

on the peaks of mice activity observed in the time course (Fig. 2B): acclimation (i.e., 0-1 h), night 1 (i.e., 5-8 h) and night 3 (i.e., 53-56 h). Statistical analysis and graphic representation of data were performed with Prism (GraphPad Software) and R (RCore Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>). Normality was assessed using the Shapiro-Wilk test (Supplementary Table 1). Given the high prevalence of non-normal distributions, the nonparametric Mann-Whitney (Wilcoxon) U test was chosen for analysis (all p -values are provided in Supplementary Table 1). A Bonferroni correction on 41 variables and 3 time-bins (adjusted significance threshold of $p < 0.00041$) is provided in Supplementary Table 1. PCA was computed using FactoMineR package (Lê et al., 2008) with R to assess genotype (Fig. 11) and cage effect (Supplementary Fig. 1). Ellipses represent the barycenter and 95% confidence interval per group. The following behavioral variables were included in the PCA: distance traveled, center time, rearing, total contacts, S-S contacts, N-AG contact, N-N contacts, train of 2, move in contact, move isolated, stop in contact, stop isolated, group of 2 and group of 3. The animal illustrations featured in the graphs were created using BioRender (URL <https://www.biorender.com>).

2.6. Code availability

Newly created scripts available on GitLab (https://gitlab.com/chavis_manzoni_lab/lmt-scripts).

3. Results

3.1. Normal day/night rhythm in groups of *Fmr1* KO mice

Activity in our group of mice was monitored across alternating light and dark phases (Fig. 2). Similar temporal dynamics of locomotor activity were observed in both genotypes (Fig. 2B). There was a significant surge in locomotor activity within the initial hour during the initial phase of the experiment when mice were first introduced to the new environment for acclimation. This heightened activity gradually diminished over the following three hours leading up to the onset of the first dark phase. Throughout all observed dark cycles, a uniform activity rhythm became evident. Over these 12-h periods, there was a noticeable spike in activity during the initial 4 h, followed by a 4-h period of reduced activity, and finally, a secondary activity peak (although smaller than the initial one) in the last 4 h of the night. As expected,

daytime cycles were characterized by low levels of mouse activity, occasionally punctuated by minor activity spikes (Fig. 2B). Based on the time course of motor activity, we selected three specific time points for further in-depth quantitative and qualitative analysis of exploratory and social behaviors. These time points corresponded to the highest peaks of activity, representing periods during which exploratory behavior and active social interaction among cagemates were most pronounced.

Specifically, these time points coincided with 1/ the peak of activity during the first hour in the arena (i.e., 0-1 h, Fig. 2B); 2/ the peak of activity during the first 4 h of the first dark period (i.e., 5-8 h, Fig. 2B) and 3/ the peak of activity during the first 4 h of the last dark period (i.e., 53-56 h, Fig. 2B).

3.2. Abnormal explorative behavior in *Fmr1* KO mice

When analyzing the distance traveled per hour in *Fmr1* KO mice, an initial hyperlocomotive phenotype is observed, which gradually normalizes over time. This heightened locomotion in *Fmr1* KO mice, in comparison to the control group, is especially pronounced during the acclimation phase in a new environment and the subsequent first period of darkness. However, during the final night spent in the same environment, both genotypes exhibit similar levels of locomotor activity (Fig. 3A). This observation is consistent with the notion of a hyperactive phenotype in *Fmr1* KO mice when exposed to an unfamiliar environment.

In the natural behavior of rodents, they tend to avoid open spaces to evade potential predators. The time spent in the center of the arena serves as a general indicator of the mouse's anxiety level. In parallel with their hyperactive locomotion phenotype, *Fmr1* KO mice spend a higher amount of time in the center during the initial phases of the experiment, while this parameter aligns more closely with the behavior of control mice during later phases (Fig. 3B, C).

Rearing, an essential element of exploratory behavior that indicates the mouse's search phase during its interaction with the environment, was also extracted (Fig. 4). Interestingly, evaluation of the total time spent in rearing, showed that *Fmr1* KO mice exhibited a deficit in the late stage (third dark period) of the experiment, but not in the early stages (acclimation or first dark period, Fig. 4C). This contrasts with the locomotor and center exploration abnormalities that were primarily evident in the early stages (Fig. 3). The altered rearing behavior in *Fmr1* KO mice during the late stage can be attributed to both a lower frequency and a shorter average duration of this behavior (Fig. 4A, B). To investigate the influence of conspecifics, rearing behavior in contact of a

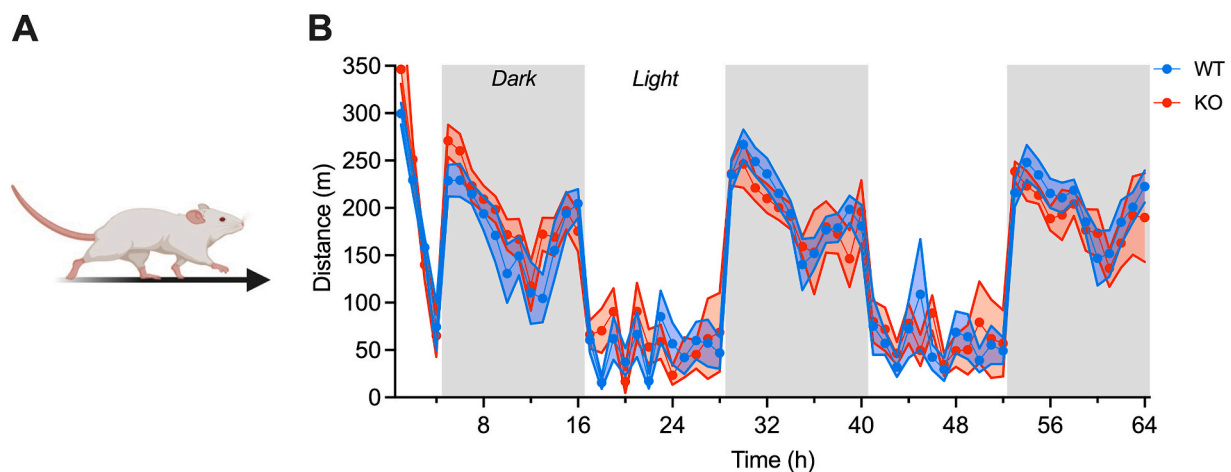


Fig. 2. Locomotor activity day/night temporal dynamics are normal in *Fmr1* KO mice. A. Illustration of a mouse in motion. B. The temporal dynamics of activity, measured as distance traveled, traced a similar day/night rhythm between *Fmr1* WT and KO mice. Single dot represents group mean value at that current step. Data are shown as mean \pm 95% CI in XY plot. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

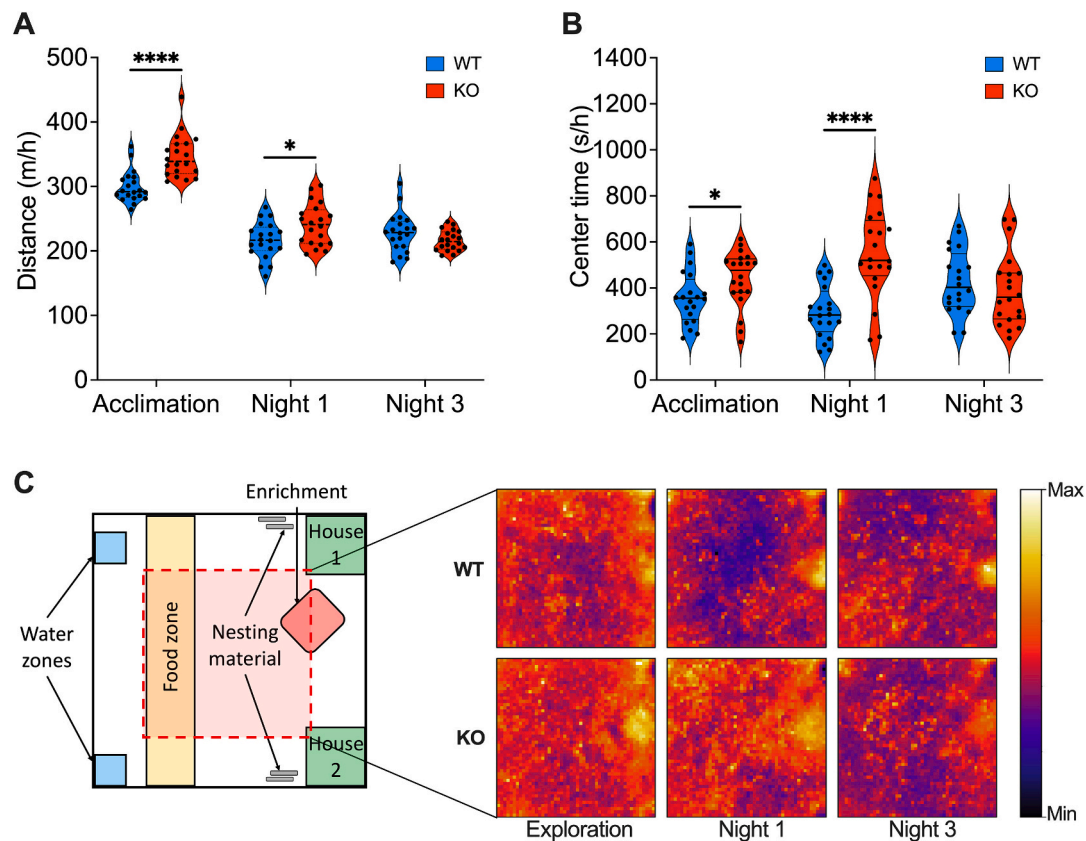


Fig. 3. *Fmr1* KO mice exhibit increased locomotion and a preference for exploring the center of the arena. **A.** *Fmr1* KO mice display hyperactivity during the acclimation and the first dark phases. **B.** During these same phases, *Fmr1* KO mice spend more time in the center of the arena compared to the controls. **C.** The average time spent per hour in the center of the arena by each experimental group was transformed into a heatmap for each analyzed period. **A-B.** Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney *U* tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cage mate (Fig. 4H) and during isolated rearing (Fig. 4D) were analyzed. During the final night, both the average (Fig. 4F, J) and total (Fig. 4G, K) duration of both behaviors was reduced in *Fmr1* KO mice. During the first night only “rearing in contact” was reduced (Fig. 4K). Furthermore, the frequency of isolated rearing did not display statistically significant differences in any of the phases (Fig. 4E), while rearing in contact was less frequent during the third night (Fig. 4I). None of the forms of rearing exhibited alterations during the acclimation to the new environment (Fig. 4). *Fmr1* KO mice exhibit impaired rearing behavior, particularly in the later stages of the experiment, with more pronounced alterations observed in rearing in contact as compared to isolated rearing.

3.3. Abnormal social interactions in *Fmr1* KO mice

Impairments in social interactions are a prominent characteristic of the behavioral patterns observed in ASD mouse models, including FXS (Bey and Jiang, 2014). The examination of continuous group behaviors over extended durations has been relatively scarce in previous research. Thus, we identified, compared, and analyzed various facets of social interaction within our mouse groups. First, we examined how *Fmr1* KO mice engaged in and maintained physical contact with their cage mates across various experimental phases. Surprisingly, during the acclimation to the new environment, *Fmr1* KO mice spent significantly more time in physical contact compared to control mice (Fig. 5A). Additionally, during the acclimation and the first dark period, we observed abnormalities in the pattern of physical contact. In these phases, *Fmr1* KO mice engaged in physical contact significantly more frequently but for shorter durations compared to controls (Fig. 5B, C).

In summary, when placed in an unfamiliar environment, *Fmr1* KO mice displayed social abnormalities characterized by an increased tendency for physical contact. This contact was more frequent but less sustained compared to controls. However, as the mice became accustomed to the new environment, this behavior normalized and returned to levels similar to the control group.

Considering the observed changes in how *Fmr1* KO mice initiate and maintain social contact, their physical interactions were categorized according to distinct engagement mechanisms. Physical contact among mice can occur in various ways, including side-by-side contacts where two mice are positioned either facing in the same direction (S-S) (Fig. 6A) or opposite directions (S-S opposite) (Fig. 6E). During the acclimation to a new environment, *Fmr1* KO mice spent a cumulative amount of time engaged in S-S and S-S opposite contacts that was comparable to controls (Fig. 6B, F). However, these contacts exhibited notable qualitative differences: S-S contacts were more frequent (Fig. 6C) but of shorter duration (Fig. 6D) in *Fmr1* KO mice compared to WT mice. Meanwhile, S-S opposite contacts during the same period were more frequent in *Fmr1* KO mice (Fig. 6G) without statistically significant differences in their average duration ($p = 0.0524$) (Fig. 6H). Additionally, a shorter mean duration of S-S contacts in *Fmr1* KO mice was also observed during the first night (Fig. 6D).

Conversely, during the last analyzed period (third night), *Fmr1* KO mice spent more time engaging in both types of contacts (Fig. 6B, F). These contacts displayed a normal frequency (Fig. 6C, G) but longer average duration compared to controls (Fig. 6D, H).

These results substantiate the notion that *Fmr1* KO mice exhibit nuanced anomalies in their engagement in side-by-side physical contact,

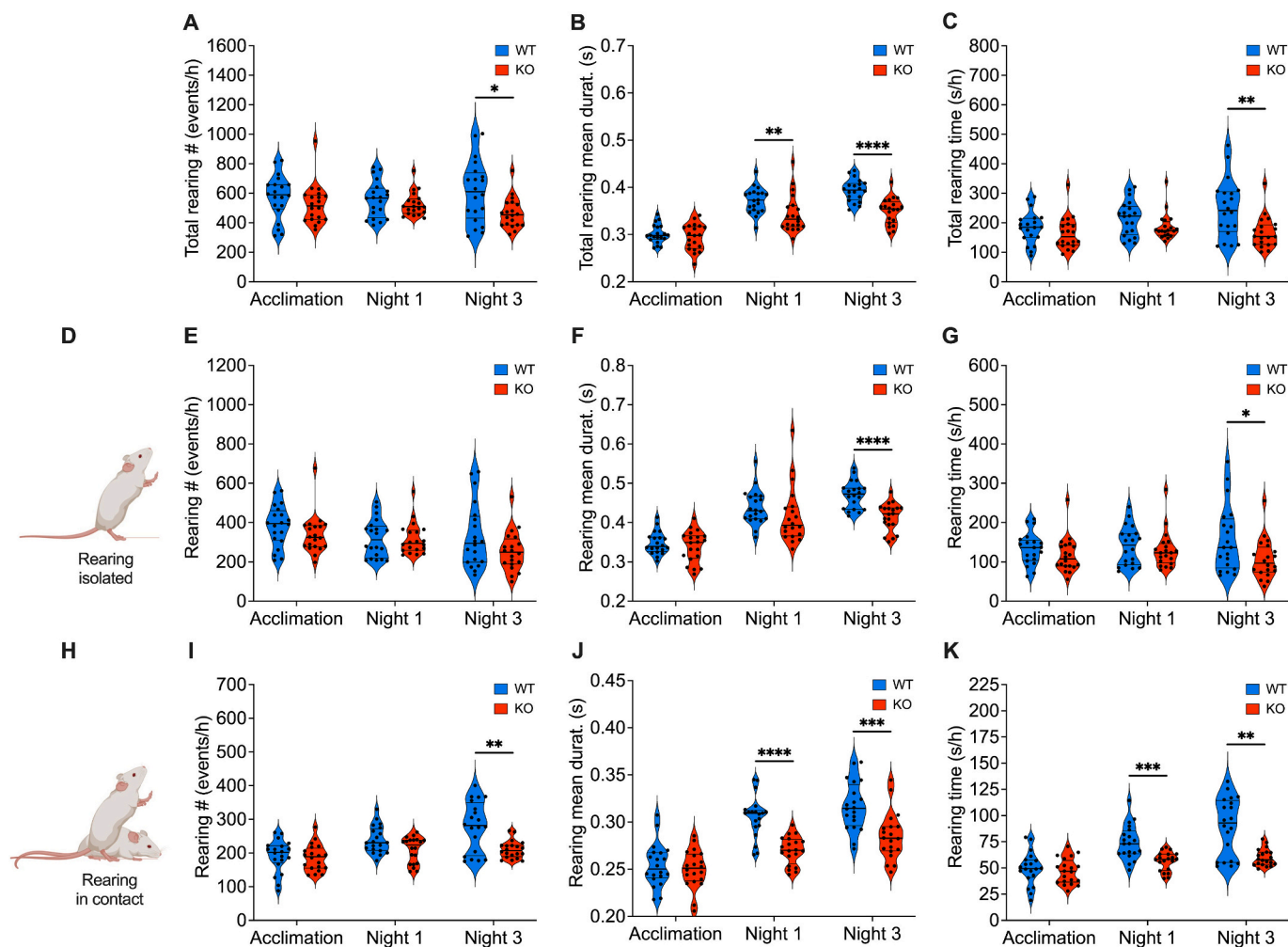


Fig. 4. Rearing behavior in Fmr1 KO mice is deficient. A-C. Compared to controls, Fmr1 KO mice exhibit less frequent rearing during the third night (A), with shorter mean duration the first and third night (B) and less total duration the third night (C). D. Illustration of rearing isolated behavior. E-G. Rearing isolated frequency is similar between genotypes, while its average (F) and total (G) duration are shorter in KO mice than WTs during the third night. H. Illustration of rearing in contact behavior. I-K. Compared to controls, Fmr1 KO mice show less frequent rearing in contact during the third night (I), with shorter average (J) and total (K) duration during the first and third night. Data are shown as violin plot with median and quartiles. Mann-Whitney *U* tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

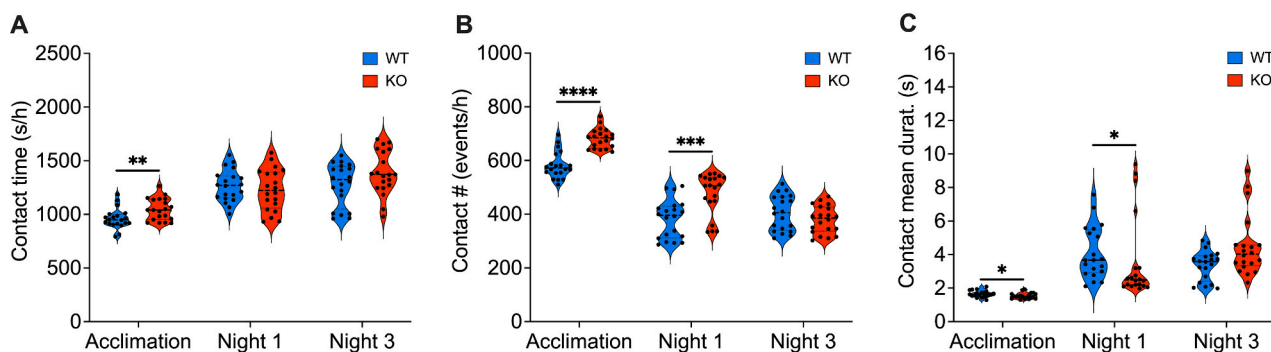


Fig. 5. Abnormal physical contact in Fmr1 KO mice. A. Fmr1 KO mice, compared to controls, display a longer overall duration of physical contacts during the acclimation. B, C. Physical contacts are more frequent (B) but shorter (C) in Fmr1 KO mice compared to WTs, during the acclimation and first night. A-C. Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney *U* tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

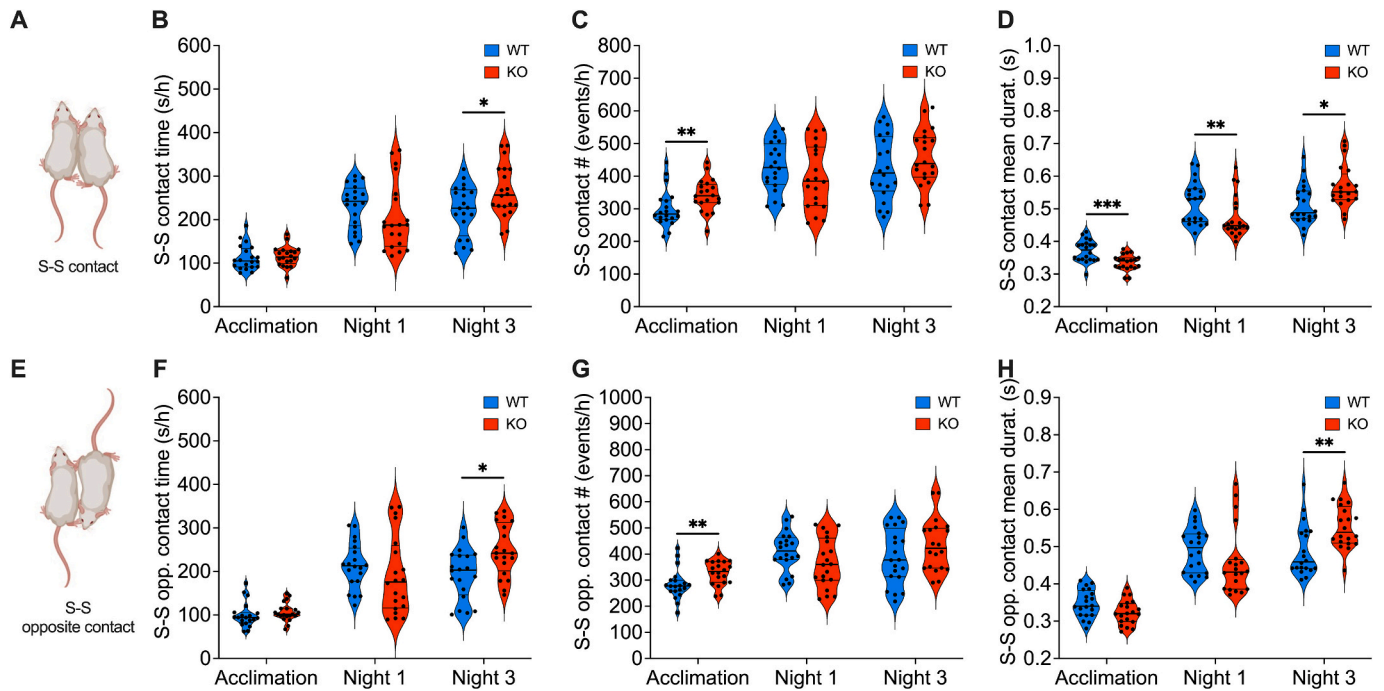


Fig. 6. The pattern of side-by-side contacts is changed in *Fmr1* KO mice. A. Illustration of 2 mice oriented in the same direction in S-S contact. B. *Fmr1* KO mice spend more time in S-S contact than controls exclusively during the third night. C. *Fmr1* KO mice have more S-S contacts than controls exclusively during the acclimation phase. D. The mean duration of S-S contacts in *Fmr1* KO mice is shorter than that observed in controls during the acclimation and the first night, but it becomes longer during the third night. E. Illustration of 2 mice oriented in opposite direction in S-S opposite contact. F. *Fmr1* KO mice spend more time in S-S opposite contact than controls exclusively during the third night. G. *Fmr1* KO mice have more S-S opposite contacts than controls exclusively during the acclimation phase. H. The mean duration of S-S opposite contacts of *Fmr1* KO mice is longer than that in controls exclusively during the third night. B-D, F-H. Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney *U* tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with the nature of these anomalies varying based on their familiarity with the environment.

Sniffing in mice plays a crucial role as a form of communication and information gathering (Arakawa et al., 2008), making it a vital component of social hierarchy and recognition (Arakawa et al., 2008; Kerckmar et al., 2011). In mice, sniffing is remarkably dynamic and is subject to variations depending on the context of their behavior. It is strongly influenced by both olfactory and non-olfactory stimuli (Wesson, 2013). Furthermore, mice adjust their sniffing frequency in response to different social situations, with reciprocal sniffing behavior aiding in the establishment and maintenance of social hierarchies within a group of mice (Wesson, 2013). In essence, sniffing behavior equips mice with the ability to navigate complex social interactions and effectively sustain their social relationships.

We examined two distinct forms of sniffing-based contacts: nose-to-anogenital (N-AG) (Fig. 7A) and nose-to-nose (N-N) (Fig. 7E). During the acclimation to the new environment, *Fmr1* KO mice indeed devoted more time to engaging in N-AG contact compared to control mice (Fig. 7B). These N-AG contacts were notably more frequent, while the average duration remained similar between the two groups. In contrast, during the final phase of the experiment (i.e., the third night), the total duration of N-AG interaction was higher in *Fmr1* KO mice than in controls (Fig. 7B) due to a longer mean duration (Fig. 7D) rather than a higher frequency of these events (Fig. 7C). *Fmr1* KO mice displayed similar alterations in N-N interaction as observed in N-AG interaction during the final night (Fig. 7F-H). However, during the acclimation phase, N-N interaction showed a comparable total duration between the groups (Fig. 7F), because of a higher frequency (Fig. 7G) and shorter duration (Fig. 7H) of these events in *Fmr1* KO mice compared to controls. Intriguingly, during the first night, *Fmr1* KO mice exhibited typical sniffing-based contacts (Fig. 7B-D, F-H). Usually, social interaction among mice involves a sequence of N-N and N-AG contacts (Fig. 7I).

Here, *Fmr1* KO mice displayed abnormal frequencies of transitions from N-N to N-AG (Fig. 7J), while the reverse transitions occurred at a similar rate to controls (Fig. 7K). Specifically, compared to controls, *Fmr1* KO mice had fewer transitions from N-N to N-AG during the initial dark phase spent in the arena. Conversely, 48 h later, the opposite pattern emerged, with these mice displaying a higher number of N-N to N-AG transitions (Fig. 7J).

In summary, the social interactions of *Fmr1* KO mice exhibit phase-dependent alterations in the initiation and maintenance of sniffing-based contacts.

When two moving mice engage in an N-AG interaction, it is referred to as a “train” contact (Fig. 8A). This behavior combines elements of social interaction (N-AG sniffing) and exploration (movement). Throughout the analyzed periods, this train movement primarily involves only two mice at a time (Fig. 8B).

In contrast to the overall N-AG contact observations, the “train of 2” behavior shows a longer total duration in *Fmr1* KO mice compared to controls, specifically during the acclimation to the new environment (Fig. 8C). This anomaly can be attributed to a higher frequency of engagement in this contact (Fig. 8D), while the average duration remains the same between the two groups (Fig. 8E).

These results suggest that in an unfamiliar environment, *Fmr1* KO mice tend to move more closely behind one another compared to controls.

3.4. The familiarity of the environment determines whether *Fmr1* KO mice move in groups or independently

The anomalies observed so far support the hypothesis that *Fmr1* KO mice tend to favor exploring a new environment in groups. To test this hypothesis, we assessed their inclination toward either moving in contact with another mouse or moving alone during different phases of the

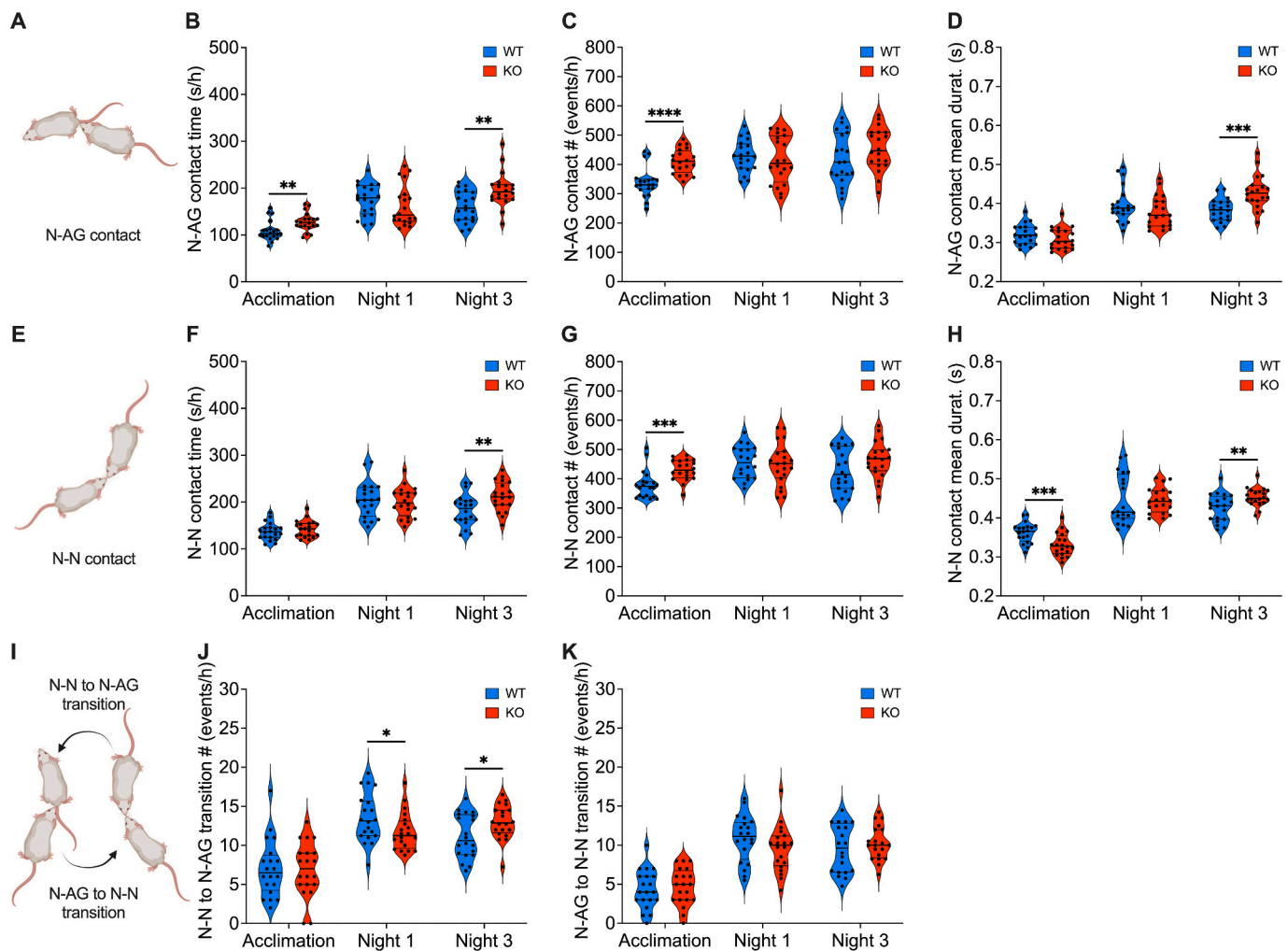


Fig. 7. Social sniffing sequences are altered in Fmr1 KO mice. A. Illustration of N-AG contact in mice. B. Fmr1 KO mice spend more time in N-AG contact during the acclimation and the third night. C. N-AG contact frequency is higher for Fmr1 KO mice during the acclimation. D. The mean duration of N-AG contact of KO is longer compared to controls during the third night. E. Illustration of N-N contact in mice. F. Fmr1 KO mice spend more time in N-N contact during the third night. G. N-N contact frequency is higher for Fmr1 KO mice during the acclimation. H. The mean duration of N-N contact of Fmr1 KO mice is shorter than controls during the acclimation, but it becomes longer during the third night. I. Illustration of N-N to N-AG transition and vice versa in mice. J. Fmr1 KO mice display more and less N-N to N-AG transitions than controls during the first and third night respectively. K. N-AG to N-N transitions were comparable between groups. B-D, F-H, J, K. Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney *U* tests. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiment (Fig. 9). During the acclimation phase and the first dark period in the new environment, *Fmr1* KO mice indeed dedicated more time to moving while in contact with a cage mate compared to controls. However, during the third dark period, this behavior returned to normal (Fig. 9B), and no significant differences were noted in the time spent not moving in contact with another mouse (Fig. 9D). Conversely, the cumulative time spent moving alone was higher in *Fmr1* KO mice only during the first night (Fig. 9F), while the time spent not moving alone was significantly lower in *Fmr1* KO mice compared to controls, particularly during the acclimation and the last night (Fig. 9H). *Fmr1* KO mice displayed a stronger preference than the control group for moving in contact with others, especially during the acclimation to the new environment (Fig. 9I). In contrast, when it comes to phases of non-movement, KO mice exhibited a greater preference for physical contact only during the last dark period (Fig. 9J).

These findings indicate that in an unfamiliar environment, *Fmr1* KO mice are less likely to explore alone compared to controls. As the environment becomes more familiar, they are more willing to be alone while exploring, but this preference disappears when they stop.

3.5. Group dynamics are different in *Fmr1* KO mice

Group dynamics are influenced by the individual social skills of their members, and changes in the social interaction patterns of *Fmr1* KO mice can have an impact on these dynamics. Consequently, an analysis of group formation parameters was carried out. In a group consisting of four individuals of the same sex, mice from both genotypes tended to spend most of their social contact time in pairs (approximately 69%), followed by groups of three (around 29%), with instances of being in groups of four being quite rare (below 2%) (Fig. 10A, B). Therefore, the analysis primarily focused on events involving the formation of groups composed of two or three mice.

In comparison to the control group, *Fmr1* KO mice spent an equivalent amount of time in pairs throughout all the phases analyzed (Fig. 10C). However, during the acclimation phase, they allocated more time to being in groups of three (Fig. 10F). Furthermore, the formation of groups of both sizes occurred more frequently (Fig. 10D, G) and had shorter durations (Fig. 10E, H) among *Fmr1* KO mice compared to controls, but only during the acclimation to the new environment and

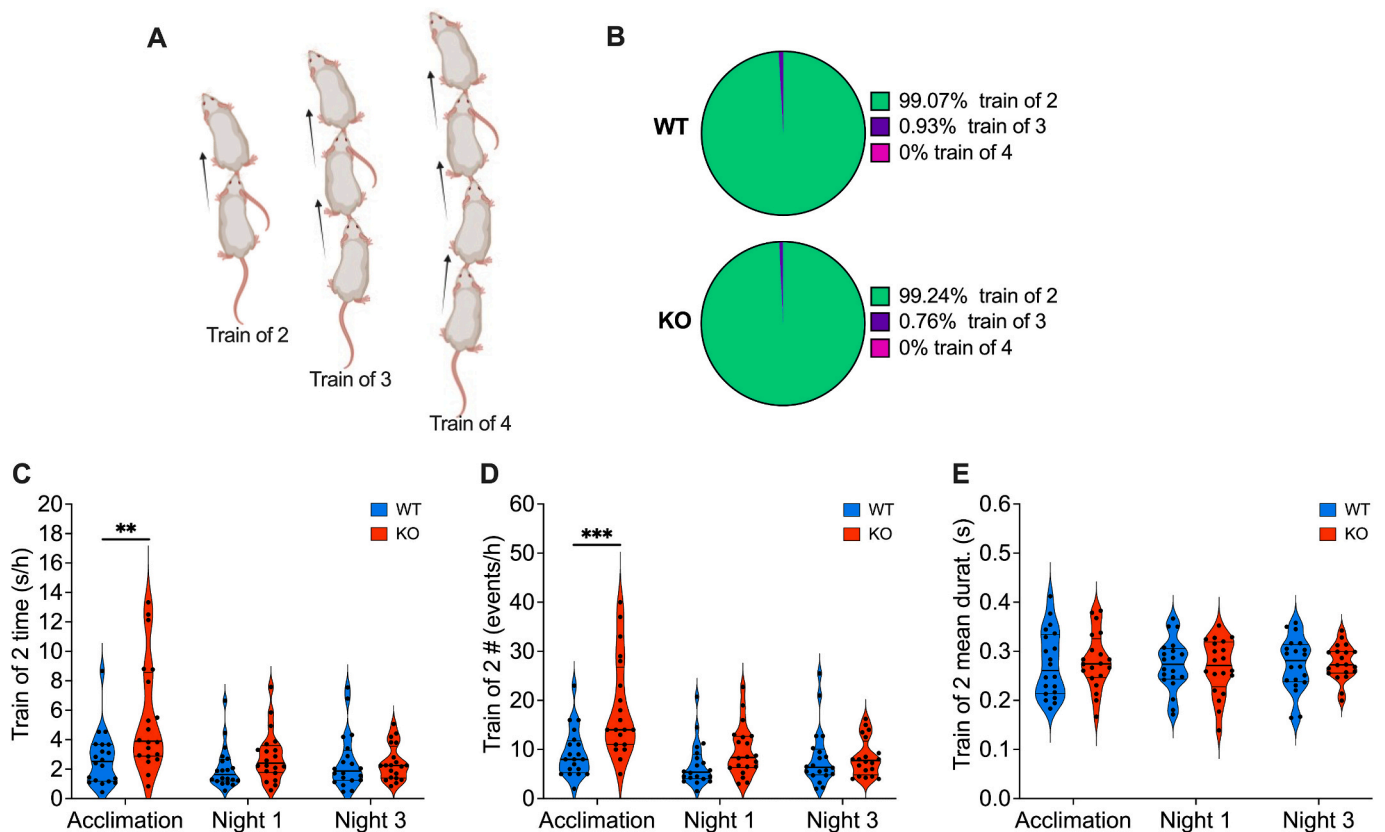


Fig. 8. *Fmr1* KO mice move more in “train” when exploring a new environment. A. Train contacts illustration with 2, 3 or 4 mice involved. B. In both genotypes, train contacts involve almost always 2 mice at a time. Pie graphs: percentages of train of 2, 3 or 4 mice, in *Fmr1* WT and KO groups (total time spent in train behavior over the three analyzed periods by each group). C–E. *Fmr1* KO mice, compared to controls, spend more time moving in a train of 2 during the acclimation phase (C), where they engage more frequently in this type of contact (D) but with the same mean duration (E). Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney *U* tests. ** = $p < 0.01$; *** = $p < 0.001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the first dark period spent within it.

In summary, in a novel environment, *Fmr1* KO mice display a significantly higher frequency of forming and dissolving social groups, which happens more rapidly than in controls. This phenotype normalizes when the environment becomes familiar.

In conclusion, in line with most of the previously presented data, the PCA conducted on the exploratory and social phenotype in *Fmr1* KO mice (see Fig. 11) corroborates that the most notable distinctions between genotypes were evident when the mice were initially exposed to an unfamiliar environment (Fig. 11A). Intriguingly, the phenotype progressively returned to levels like those of the control group as the mice became more accustomed to their surroundings over time (Fig. 11C).

4. Discussion

In this study, we compared the continuous, unsupervised, and spontaneous social interactions between groups of WT and *Fmr1* KO mice to understand the dynamics of social behavior in FXS. The findings reveal complex abnormalities in the exploratory and social behavior of *Fmr1* KO mice, with the most significant differences occurring during their initial adjustment to a new environment. Over time, these altered behaviors gradually decreased and approached those of WT mice. This dynamic FXS phenotype is characterized by heightened activity, a greater inclination to explore exposed areas, increased interest in social interaction, and a distinctive pattern of social interactions.

The findings indicate that groups of *Fmr1* KO and WT mice exhibited a comparable day/night rhythm in locomotor activity, consistent with

previous research on individually housed *Fmr1* tm1Cgr mice (e.g., a 25-h experiment by (Pietro Paolo et al., 2011), and a 16-day observation by (Bonasera et al., 2017)). While sleep problems have been observed in humans with FXS (Kronk et al., 2010; Budimirovic et al., 2022), and alterations in circadian clock-related parameters have been noted in the *Drosophila* model of FXS (Zhao et al., 2021; Dockendorff et al., 2002), it appears that these issues do not have apparent effects on the locomotor activity rhythms of mice in day/night cycles.

In this study, we observed that *Fmr1* KO mice displayed hyperactivity during the initial phases of the experiment when they were active, but not during the later stages. This hyperactive behavior appeared to be induced by exposure to an unfamiliar environment. It is worth noting that individuals with FXS often exhibit symptoms of hyperactivity (Davidson et al., 2022). Consistent evidence points to a hyperactive locomotor phenotype in both *Fmr1* KO and *Fmr1* tm1Cgr mice when assessed using conventional tests like the open field (Gaudissard et al., 2017; Jung et al., 2012; Liu et al., 2011; Saré et al., 2016; Ding et al., 2014; Dolan et al., 2013; Gurney et al., 2017; Peier et al., 2000; Spencer et al., 2005; Gholizadeh et al., 2014; Mineur et al., 2002). However, these tests, which usually last for 30 to 120 min, are not optimal for gaining a comprehensive understanding of the novelty-dependent aspects of this behavior. Moreover, a study documented a hypoactive locomotor phenotype in *Fmr1* tm1Cgr mice for 16 days after a 5-day habituation period in their familiar environment (Bonasera et al., 2017). Thus, FMRP-deficient mice may transition between hyperactive and hypoactive phenotypes depending on the level of environmental familiarity.

An alternative explanation for our findings is that the elevated

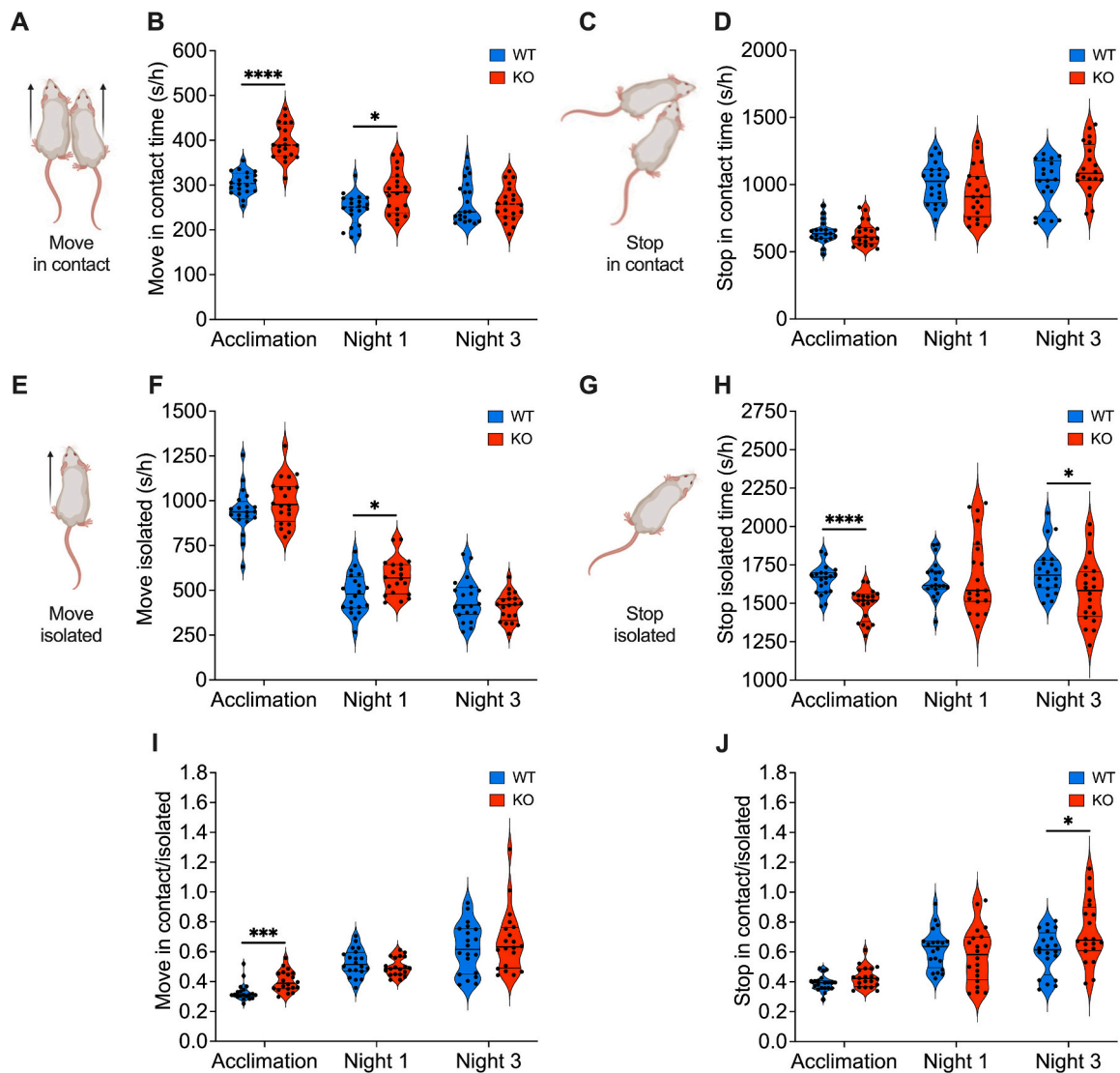


Fig. 9. *Fmr1* KO mice show elevated preference for social contact while moving or not, depending on the familiarity with the environment. A. Illustration of two mice moving in contact. B. *Fmr1* KO mice spend more time moving in contact than controls specifically during acclimation and first night. C. Illustration of two stationary mice in contact. D. The time spent in contact during non-moving phases is similar between groups. E. Illustration of a mouse moving alone. F. *Fmr1* KO mice spend more time than WTs in moving isolated during the first dark period. G. Illustration of a stationary isolated mouse. H. *Fmr1* KO mice spend less time alone when they do not move during the acclimation and last dark period. I, J. *Fmr1* KO mice, compared to controls, have higher preference for contact while moving during acclimation (I) and, conversely, while stationary during the last night (J). A, B, D, F, H, I, J. Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney U tests. * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

locomotor activity observed in *Fmr1* KO mice during the initial experimental phases may be driven by an anxious state, triggered by a hyperarousal response to environmental novelty. This interpretation is consistent with the phenotypic characteristics of individuals with FXS, who often exhibit increased motor activity and anxiety in novel, unfamiliar situations, which is thought to be mediated by autonomic hyperarousal (Cohen, 1995). If this is the case, the decrease in locomotor activity observed later in the experiment may be attributed to habituation to the environment and reduced anxiety, rather than a shift in the underlying hyperactive phenotype. This perspective underscores the significance of considering anxiety as a contributing factor to the behavioral responses of mice and individuals with FXS, and its potential impact on the manifestation of hyperactivity.

Individuals with FXS are frequently diagnosed with anxiety disorders (Cordeiro et al., 2011). Convergent evidence from mouse models of FXS demonstrates anxiety-related alterations in test settings such as the open field (Saré et al., 2016; Peier et al., 2000; Spencer et al., 2005; Liu et al.,

2011; Yuskaitis et al., 2010) and the elevated plus/zero maze (Liu and Smith, 2009; Gaudissard et al., 2017; Jung et al., 2012; Saré et al., 2016; Yuskaitis et al., 2010; Chen et al., 2013; Heulens et al., 2012). The anxiety state of mice is typically assessed in these tests, based on the premise that a rodent preferring open and exposed spaces exhibits lower anxiety levels. Paradoxically, despite the anxious nature of the FXS phenotype, mouse models of FXS consistently display a preference for open spaces in these tests. This contradictory aspect of the FXS mouse models remains unexplained. In our study, we observed that *Fmr1* KO mice spent more time in the center of the arena than controls during the early stages, but not in the late stages, consistent with previous literature. While this endophenotype may be a limitation of the murine model, it is also possible that the increased time spent in open spaces results from other aspects of the pathology, rather than a mere reduction in physiological anxiety. For instance, the significant intellectual disability caused by this mutation may alter the risk perception of open spaces, making them non-anxiogenic for FXS murine models.

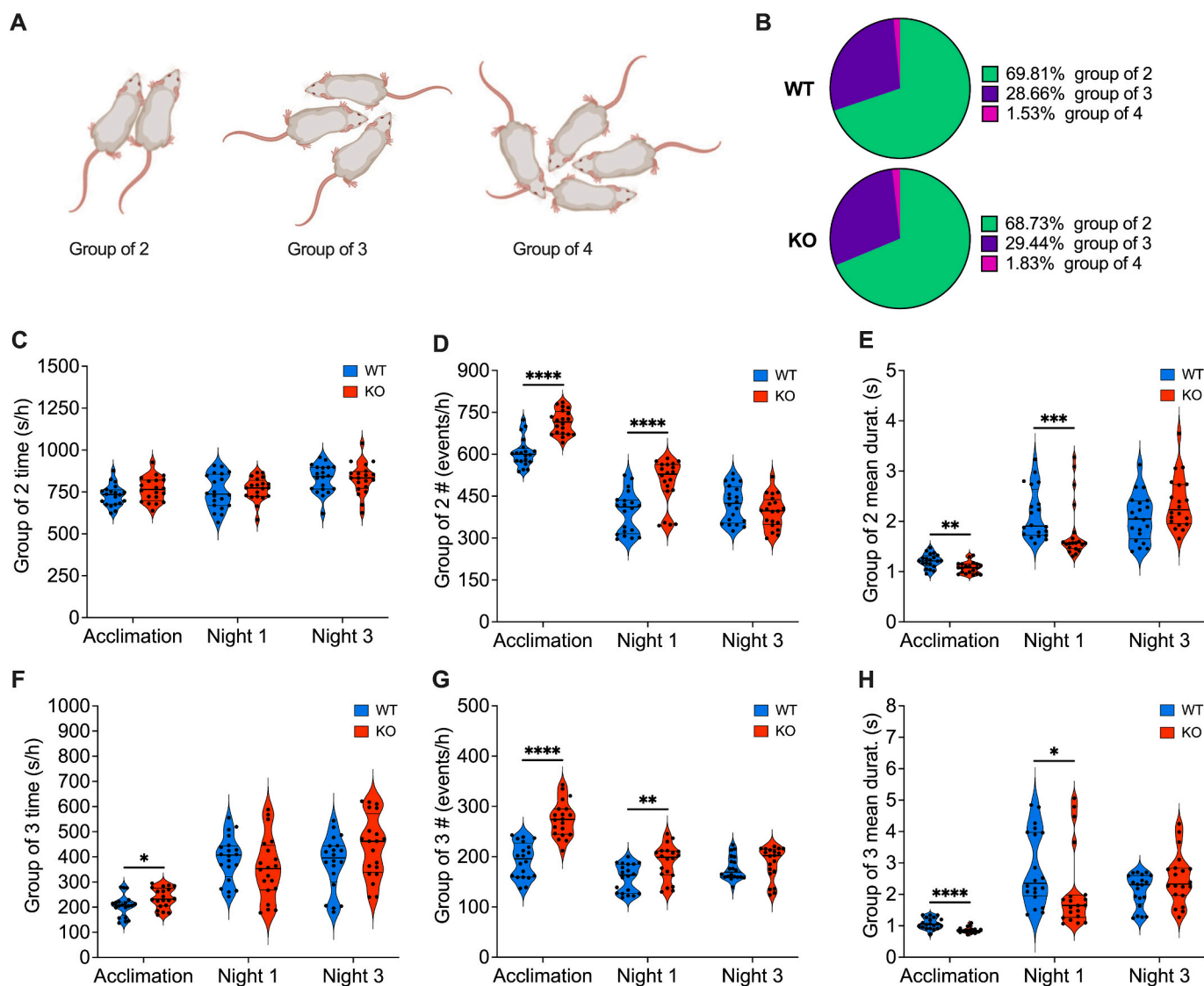


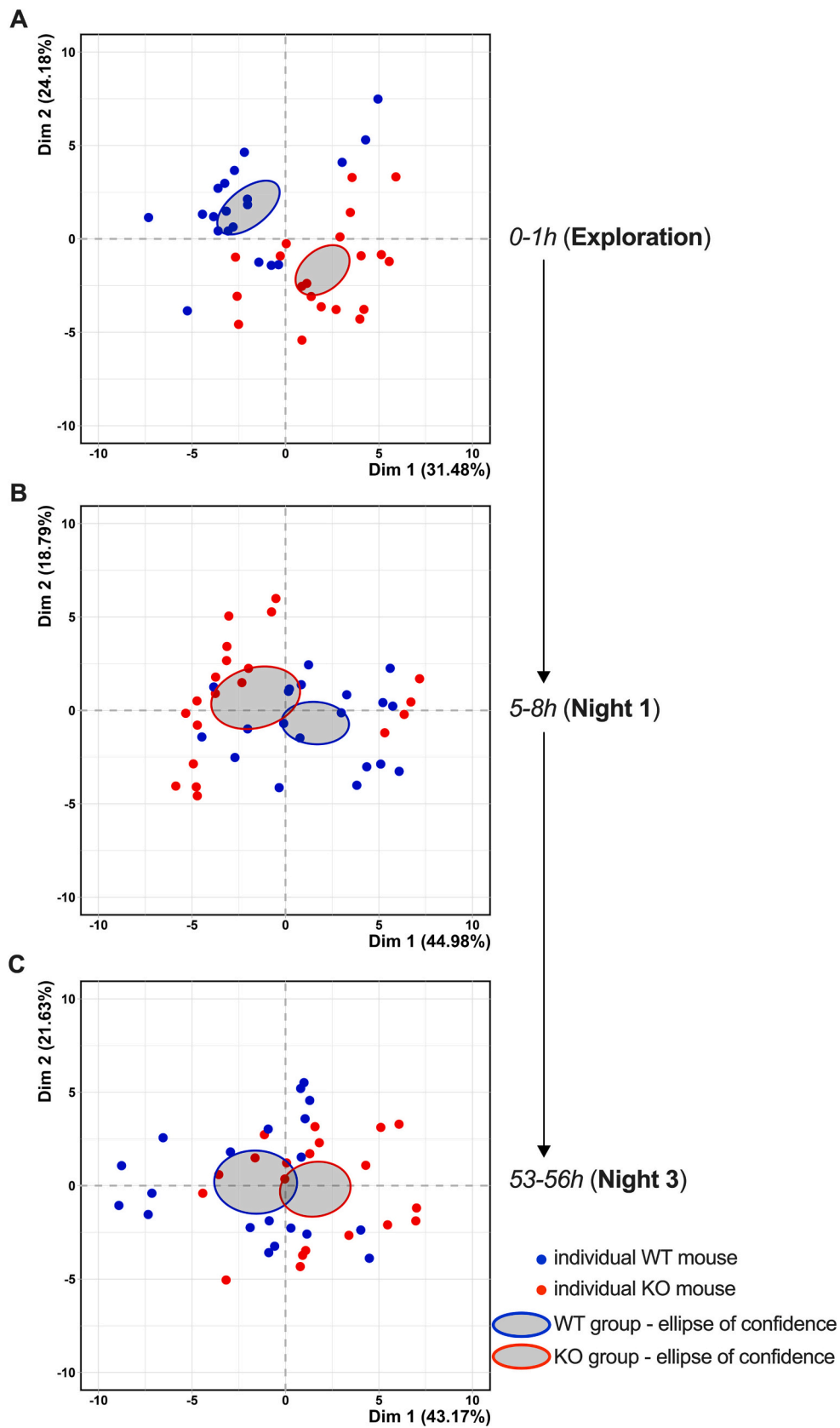
Fig. 10. *Fmr1* KO mice show abnormal group dynamics depending on the familiarity of the environment. A. Illustration of groups of 2, 3 or 4 mice. B. Mice of both genotypes predominantly engage in social interactions in pairs (~69%), followed by groups of three (~29%), and rarely in groups of four (below 2%). Pie graphs: percentages of group of 2, 3 or 4 mice for *Fmr1* WT and KO mice (total time spent in group over the three analyzed periods by each genotype). C-E. The total time spent in pairs does not vary between genotypes (C), although *Fmr1* KO mice form pairs more frequently (D) and with shorter durations (E) during acclimation and the first night. F-H. During acclimation, groups of 3 *Fmr1* KO mice lasted longer overall, were more numerous (G), but each individual mouse had shorter durations (H). C-H. Single dot represents an individual mouse. Data are shown as violin plot with median and quartiles. Mann-Whitney U tests. * = $p < 0.05$; ** = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$. WT group in blue, $N = 20$; KO group in orange, $N = 20$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Additionally, the concurrent locomotor hyperactivity and increased exploration in *Fmr1* KO mice may drive their exploration of open spaces, as suggested by (Melancia and Trezza, 2018). Therefore, a lower perception of open spaces as an anxious stimulus and a greater drive for exploration may underlie this behavioral phenotype. Furthermore, other behaviors, such as moving closely behind each other, suggest an anxious or discomforted state in KO mice in novel environments, highlighting the complexity of their anxiety-related behaviors.

Social deficits are a hallmark of ASD and FXS (Cregenzán-Royo et al., 2022), with FXS characterized by social anxiety and aversive perception of gaze, without the prominent reduction in social interest seen in non-syndromic ASD (Hong et al., 2019; Roberts et al., 2018). In our study, *Fmr1* KO mice exhibited an increased total time spent in physical contact with a cage mate during their initial adjustment to the new environment, but this behavior normalized to levels like controls in later stages. Specifically, during the acclimation phase and the initial active period (i.

e., the first dark period), social contacts were more frequent but of shorter average duration compared to controls. We hypothesize that this behavior could reflect an elevated interest or need for social contact in response to discomfort. Noteworthy, the increase in social contacts coincided with the presence of hyperactive and altered anxiety phenotypes, implying a potential influence of these traits on social skills. This observation aligns with existing research that highlights the significant impact of hyperactivity (Chromik et al., 2019) and anxiety (Hong et al., 2019) on social functioning in individuals with FXS. Furthermore, the pattern of 'frequent approaching followed by rapid disengagement' resembles an approach-avoidance conflict, as indicated by several clinical studies.

Individuals with FXS exhibit physiological hyperarousal, characterized by autonomic dysfunction and sensory sensitivity. This combination predisposes them to difficulties in regulating negative emotions, contributing to social anxiety (Crawford, 2023). Despite heightened



(caption on next page)

Fig. 11. *Fmr1* KO mice display atypical behavior upon initial exposure to an unfamiliar environment that gradually normalizes with time. A-C. Principal component analysis (PCA) of exploratory and social behavior shows an atypical phenotype of *Fmr1* KO mice during the first hour in an unfamiliar environment (A) that gradually normalizes in the later analyzed phases (B and C). PCA was performed on the behavior recorded during 0-1 h, 5-8 h, 53-56 h time intervals. Behaviors included in PCA are: distance traveled, center time, rearing, total contacts, S-S contacts, N-AG contact, N-N contacts, train of 2, move in contact, move isolated, stop in contact, stop isolated, group of 2 and group of 3. Single dot represents an individual mouse. Ellipses represent the barycenter and confidence interval per group. Data are shown as PCA of individuals, dimension 1 and 2 percentages of variance are displayed in graphs. WT group in blue, N = 20; KO group in orange, N = 20. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

social anxiety and avoidance tendencies, there is still a strong social preference and interest in individuals with FXS that exhibit behaviors suggesting their willingness or desire to interact with others (Cohen et al., 1988; Hong et al., 2019; Bailey et al., 1998; Cornish et al., 2008; Crawford et al., 2020; Guy et al., 2020).

In this comprehensive examination of the FXS phenotype in groups of mice, the most notable disparities between genotypes emerged during the initial exposure to an unfamiliar environment. Subsequently, selected phenotypes exhibited a gradual normalization, converging toward levels comparable to those of the control group as the mice became more acclimated to their surroundings. This observation aligns with heightened sensitivity traits, including increased arousal (Cohen, 1995), sensory hypersensitivity (Rais et al., 2018), and reduced predictive capabilities (Sinha et al., 2014), often observed in individuals with FXS. These traits lead to an enhanced perception of novelty in environmental stimuli. Consequently, encountering uncomfortable situations, such as unfamiliar environments, can potentially trigger or exacerbate behavioral abnormalities in FXS individuals.

5. Conclusion

In conclusion, our study showcases the potential of the LMT to reveal the dynamic adaptive behavior of *Fmr1* KO mice in unfamiliar environments. The LMT offers a significant advantage over current approaches, providing a more ecologically valid and human-like model of behavior. This innovative tool may prove invaluable in pre-clinical medication trials and translational research, ultimately informing the development of more effective therapeutic strategies for individuals with FXS. Furthermore, elucidating the neural mechanisms underlying this dynamic phenomenon may have significant implications for the development of innovative therapeutic approaches, including both behavioral interventions and pharmacological treatments.

6. Limitations of the study

This study has several limitations that should be considered in future research using this experimental approach to study FXS in mice. Firstly, our analysis is based on movement, trajectory, and contact data, which may oversimplify the investigation of social behavior. While nonverbal cues are important in murine communication, ultrasonic vocalizations also play a crucial role. Integrating vocalization recordings, particularly at the individual level, into future experiments would provide a more comprehensive understanding of social behavior. Secondly, our findings suggest a behavioral phenotype centered around hyperarousal in response to environmental novelty, but this hypothesis is primarily based on exploration behavior rather than direct measurements of physiological indices of autonomic arousal. Future studies should consider implementing minimally invasive measurements of electrodermal, pupillary, or cardiac responses, or alternatively, direct but more invasive measurements, such as physiological stress markers in the blood, to provide a more complete understanding of the anxiety phenotype in *Fmr1* KO mice. Additionally, incorporating these measurement approaches could serve as indicators of anxiety, offering valuable insights into the contradictory nature of FXS murine models' predisposition for anxiogenic spaces. Further research is needed to fully elucidate these complexities and refine our understanding of FXS in mice using this experimental paradigm.

Aggression is a natural behavior among male mice, particularly in

novel environments and under stressful conditions. This behavior can potentially influence social and exploratory performance, and may play a role in the observed FXS phenotype. However, to date, developing a code to reliably distinguish aggression from other behaviors (e.g., "train of 2") in this experimental setting has proven challenging. Therefore, in this study, we manually scored aggressive behavior in the early test phase to count the number of aggressive events per group. While aggression was absent in all WT groups and present in 40% of the *Fmr1* KO groups (2 out of 5 groups), no statistically significant differences were observed between genotypes (Supplementary Fig. 2). Future research will be necessary to address the limitations of this approach and systematically evaluate aggression. This includes incorporating metrics such as the duration, latency, and severity of aggressive episodes, and conducting individual analyses with larger sample sizes. These improvements will provide a clearer understanding of the potential role of aggressive behavior in the FXS socio-behavioral phenotype observed in this study.

Finally, this study analyzed the behavior of social groups composed of same-genotype mice. The genotypic homogeneity, while necessary for establishing a fundamental understanding of the core characteristics of the two phenotypes, presents significant limitations. Interacting exclusively with individuals of the same genotype may not accurately reflect certain aspects of social contexts relevant to human conditions. Additionally, the lack of genotype diversity within each cage eliminated internal controls, resulting in a significant cage effect (Supplementary Fig. 1). This must be acknowledged as an intrinsic limitation of the experimental design. Accordingly, investigating the behavioral effects of interactions between WT and KO mice will be a crucial next step. These interactions could be key environmental factors influencing the neurodevelopmental trajectories of both genotypes. Future studies on this topic could provide valuable insights, potentially highlighting the importance of behavioral therapies and environmental enrichment in enhancing skill development in patients with neurodevelopmental disorders.

Ethics statement

Animals were treated in compliance with the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health Guide for the care and use of laboratory animals. The French Ethical committee authorized this project (APAFIS#34573-202201071122121v3).

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CRediT authorship contribution statement

Gabriele Giua: Conceptualization, Data curation, Formal analysis, Validation, Writing – review & editing. **Benjamin Strauss:** Conceptualization, Data curation, Formal analysis, Validation. **Olivier Lassalle:** Validation, Methodology. **Pascale Chavis:** Conceptualization, Supervision, Methodology, Writing – review & editing. **Olivier J. Manzoni:** Conceptualization, Supervision, Funding acquisition, Methodology,

Project administration, Writing – original draft, Writing – review & editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Chat GPT 3.5 in order to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no competing interest.

Data availability

All data reported in this paper will be shared by the lead contact upon request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pnpbp.2024.111111>.

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