Behavioural Brain Research 332 (2017) 50–58

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Spectral and temporal properties of calls reveal deficits in ultrasonic vocalizations of adult *Fmr1* knockout mice

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ARTICLE INFO

Keywords:
Fragile X syndrome
FXS
Ultrasonic vocalizations
USV
Communication deficits
Scent marking

ABSTRACT

The *Fmr1* knockout (KO) mouse has commonly been used to investigate communication impairments, one of the key diagnostic symptoms observed in Fragile X syndrome (FXS) and Autism spectrum disorder (ASD). Many studies have found alterations in ultrasonic vocalizations (USVs) in neonatal *Fmr1* KO mice, however, there is limited research investigating whether these deficits continue into adulthood. In the present study, we examine differences in female urine-induced ultrasonic vocalizations, scent marking behavior, odor discrimination, and open field activity in adult male *Fmr1* KO and wildtype (WT) mice. Overall, we found extensive alterations between genotypes in both spectral and temporal properties of ultrasonic vocalizations. There was no difference in the average number of calls emitted by both genotypes, however, *Fmr1* KO mice emitted calls of a higher frequency, decreased amplitude, and shorter duration than WT mice. Spectrographic analyses revealed statistically significant differences between genotypes in the types of calls emitted. Contrastingly, we found no differences in scent marking behavior, a form of social communication, or in odor discrimination and activity levels of the mice. The results corroborate previous studies emphasizing the importance of qualitative differences observed in vocalization behavior of *Fmr1* KO mice, rather than quantitative measurements such as number of calls emitted. Overall, the study confirms the presence of abnormalities in vocalization behavior in adult *Fmr1* KO mice that we believe are consistent with communication deficits seen in the syndrome.

1. Introduction

Fragile X syndrome (FXS), an X-linked neurodevelopmental disorder, is the most common heritable cause of intellectual disability [1]. It is characterized by expansion of a CGG repeat in the 5′ untranslated region of the fragile X mental retardation gene (*FMR1*) [2,3]. This expansion ultimately results in silencing of the gene leading to an absence of fragile X mental retardation protein (FMRP) [4,5]. Deficiency of FMRP can lead to intellectual disability, behavioral and social deficits, as well as altered communicative abilities exhibited by FXS patients [6,5].

Previous research has found those with FXS to have substantial impairments in conversational speech, including poor topic maintenance, run-on sentences, and disorganized speech [7,8]. In addition, conversations are often affected by impairments in sociability and anxiety, such as inappropriate eye contact with others and gaze aversion [9,10]. Individuals with FXS also demonstrate difficulty with pragmatic skills, such as perseveration of words, sentences, or topics, which may be reflective of a type of stereotypic behavior commonly seen in the syndrome [11,12]. Furthermore, individuals with FXS have a tendency to speak in short bursts, produce stuttering-like repetition of sounds, and have variability in rate of speech [13,14]. Overall, those affected by FXS exhibit altered communicative abilities that could potentially have a negative effect on their cognition, sociability, and quality of life.

The investigation of ultrasonic vocalization (USV) deficits has been previously explored in various mouse models of neurological diseases, both in early development from postnatal day (PD) 2–14 [15–19] and in adulthood ranging from PD60 to 120 [20,21,18]. Mouse pups begin emitting USVs shortly after birth and continue, although at a reduced rate, throughout adulthood in response to specific situations. In neonatal mice, they are known to occur between frequencies of 30–90 kHz and are elicited from mice upon separation from the nest, dam, or littermates [22,23]. Numerous studies have found examinations of USVs in FXS and Autism Spectrum Disorder (ASD) mouse models to be an important parameter when investigating social communication in mouse pups, however, there have been few studies exploring USVs in adult mouse models [15–17,24,25]. USVs in adult

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http://dx.doi.org/10.1016/j.bbr.2017.05.052
Received 20 December 2016; Received in revised form 22 March 2017; Accepted 24 May 2017
Available online 26 May 2017
0166-4328/

mice have been investigated in various mating and courtship paradigms, social encounters and intruder settings, and in response to urine of female mice [23,26].

The few studies examining adult USVs in FXS focus mainly on courtship behavior [20,21]. Rotschafer et al. [21] found the number of USVs in adult male *Fmr1* KO mice to be reduced compared to wild type (WT) controls in a mating paradigm [21]. However, this study recorded USVs with both male and female mice present in the chamber. This makes it difficult to determine whether analyzed USVs consisted of solely male vocalization patterns and that the presence of a female did not confound results. In a separate study, investigators used a different courtship paradigm to induce USVs and found no difference in the number of USVs emitted by adult male *Fmr1* KO mice, but found an increase in "u" syllables, while WT littermates vocalized "h" syllables more often [20]. While the functional importance of specific syllables based on frequency and complex patterns is currently unknown, they are organized into phrases that are thought to resemble repetitive speech patterns typically found in those with the syndrome. This study removed females prior to recording, however, one caveat to both of these studies is that USVs are induced via direct exposure to the female mice. Call production has shown to be at its peak during initial exploratory activity in response to the female, thus a more ideal method would have female urine present for the entire testing period [25]. Urine from female mice has found to be sufficient to elicit USVs in male mice without the presence of the female [27].

Investigation of USV deficits, along with social behavior, by examination of male USVs in response to female urine allows calls to be directly attributed to the male and for consistent pheromone exposure throughout the paradigm. In addition, urine-induced vocalization behavior is highly dependent on previous social interaction and social status in mice [28–30,23]. Wohr et al. [18,25] found that adult BTBR T + tf/J mice, another mouse model of autism, emitted minimal USVs and scent markings in response to female urine compared to WT mice [25]. Scent markings are urinary pheromone traces deposited in strategic areas that act as an additional mode of communication. They can be produced by mice in a variety of situations, including to attract partners, demarcate territories, and recognize the reproductive state of potential mates [31]. This additional social communicative behavior has not yet been examined in *Fmr1* KO mice.

Further investigation of the communicative behavior of adult male *Fmr1* KO mice is critical in order to gain a comprehensive understanding of specific call types emitted in the context of mating. While there is evidence of alterations of USVs in postnatal *Fmr1* KO mice, few studies have examined vocalization behavior in FXS adult mice. In the present study, we investigate differences in female urine-induced USVs of male *Fmr1* KO mice compared to WT mice. Spectrographic analyses were conducted on USVs to examine both spectral and temporal variability between the calls of *Fmr1* KO and WT mice. We also examined scent marking behavior to determine whether *Fmr1* KO mice differ in how they use olfactory communicative signaling to attract mates and assert their dominance [32]. In addition, we tested overall activity levels of the mice during USV recording to identify whether hyperactivity effected vocalization behavior, as well as tested odor discrimination abilities to confirm the absence of any underlying olfactory differences. We hypothesized that *Fmr1* KO mice will exhibit altered vocalization patterns compared to WT mice, including specific call types that may be reflective of repetitive behavioral tendencies characteristic of Fragile X syndrome. We postulate that both groups of mice will produce a similar amount of scent markings. We also hypothesize *Fmr1* KO mice to exhibit hyperactivity in the open field and similar odor discriminatory abilities as WT mice.

2. Materials and methods

2.1. Animals and housing

Subject mice included adult male FVB/NJ littermates in which 19 were *Fmr1* knockout (KO) and 18 were wild type (WT) mice. All mice were generated and group housed at Baylor University in standard laboratory conditions (22 °C, 12-h light/12-h dark diurnal cycles) with food and water provided *ad libitum*. Experimental testing was performed between 12:00 and 16:30 each day during the 12-h light period. All procedures were conducted in compliance with the Baylor University Institutional Animal Care and Use Committee and the *National Institute of Health Guidelines for the Care and Use of Laboratory Animals*.

2.2. Experimental design

2.2.1. Previous female exposure

Prior to vocalization recording, male subject mice were introduced to female mice of the same FVB/NJ strain in order to control for history of social experience. One male *Fmr1* KO or WT mouse was placed with a female *Fmr1* WT mouse of similar age in a clean polycarbonate cage with bedding for 5 min, one week prior to testing. Males were not allowed to attempt copulation with females during the 5 min female exposure.The female mice were not littermates with the male subject mice.

2.2.2. Urine collection

Female urine was obtained from the *Fmr1* WT mice in estrous that testing mice were previously exposed to. In order to coordinate estrous cycles of 2–4 female mice housed together, bedding from a male FVB/NJ cage was placed in a cage of females. Estrous cycles of female mice were then visually inspected and tracked daily. A female was considered in estrous when the vaginal area was red, inflamed, and open [33]. To elicit urine dissemination from the donor female, the mouse was removed from its cage and gently stroked in an anterior to posterior direction on its abdomen. Urine was collected in a 1.7 ml Eppendorf tube. Immediately prior to testing, we used a pipette to transfer 20ul of fresh female urine onto the center of a piece of Strathmore paper (Strathmore Drawing Paper Premium, recycled, microperforated, 400 series; Strathmore Artist Papers, Neenah, WI, USA) able to effectively absorb drops of mouse urine which lined the bottom of the testing chamber.

2.2.3. Test procedure

Before testing, mice were weighed and allowed to habituate in the testing room for 30 min. Ultrasonic vocalization recordings, scent markings, and open field activity were simultaneously recorded for each subject mouse for 5 min. Mice were individually placed in an acrylic, sound-attenuating chamber (40 cm × 40 cm × 30 cm) in an isolated room controlled for temperature, light levels, and background noise. Urine-induced USVs were recorded for 5 min using a condenser ultrasonic microphone (CM16/CMPA, Avisoft Bioacoustics, Germany, part #40011) connected to an ultrasound-recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics, part #41161/41162) suspended within the testing chamber. To measure locomotion and repetitive behavior during the testing period, locomotor activity was measured with automatic optical animal detection software (Fusion by Omnitech Electronics, Inc., Columbus, OH). Total distance and time spent in the center and surround regions, stereotypy time and episode count, vertical episode count, and clockwise and counter-clockwise rotations were recorded. A map consisting of a center region (20 cm by 20 cm) was imposed, and any activity beyond this area is considered to be time spent in the surround region. Stereotypy is typically observed during grooming and consists of an animal breaking the same beam (or set of beams) repeatedly in the open field apparatus. A break in