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Modeling Social Communication Deficits in Mouse Models of Autism

Caterina Michetti, Laura Ricceri, and Maria Luisa Scattoni*

Neurotoxicology and Neuroendocrinology Section, Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Rome, Italy

Abstract

Male and female mice emit ultrasonic vocalizations during infancy when pups are separated from mother and littermates, as well as at adulthood in different experimental/social contexts. Mouse ultrasonic vocalizations had become now a popular assay for behavioral phenotyping throughout the life-span of models of autism since this response represents the best option to detect deficits within the social communication domain in the mouse species. In the present review, we describe the available methods to elicit and record mouse ultrasonic vocalizations in different social contexts and at different ages. Behavioral data collected on autism animal models in these paradigms/contexts are also discussed. Moreover, we strongly emphasized the need of a standardization of the behavioral methods to better compare results from different laboratories.

Thanks to the progresses of computer technology, researchers can now perform detailed analyses of the vocal repertoire (classifying ultrasonic vocalizations into different categories) in autism mouse models. Recently, these analyses have revealed unusual vocal patterns in selected mouse lines. This innovative approach allows to detect also qualitative alterations in the social communication repertoire usually not identified with the standard analysis of emission rate. Future studies should be aimed at performing quantitative and qualitative analyses of vocalization patterns also in preclinical studies evaluating potential treatments in validated autism mouse models.

Introduction

A long series of studies found that mice have the capability to vocalize across a broad range of frequencies that extend from as low as the human-audible range and can extend well into the ultrasound range, above the limit of human hearing (20 kHz) [1,2]. Audible squeaks are produced by laboratory mice in stressful and painful situations [3] such as during handling and restraint [4], grid-shock test [5], or during aggressive encounters [6,7]. In reproductive contexts, human-audible squeaks are produced by females when a sexually motivated male is interacting with a non receptive female [8]. Vocalizations in the ultrasonic range are emitted by adult mice in some social contexts (Figure 1) [6,9-19]. Pups separated from the nest emit calls which the parents use to locate the straying pup and retrieve it to the nest (Figure 1) [10,12,20-22].

Since their first description [22], it appeared likely that pup Ultrasonic Vocalizations (USVs) play a role in the survival of the young, particularly through mother-young relationships. Adult rodents are certainly able to hear these sounds, and the signals have at least some communication value. The function of the isolation calls was supposed to elicit retrieving responses of the mother and to guide her to the young. In support of this hypothesis, Zippelius and Schleidt [22] published their discovery that under conditions of stress, cold and hunger, the young ones of three species of myomorph rodents produced ultrasounds at least to the age when their eyes were open. Female mice retrieved live pups from outside the nest but dead or narcotized pups which could not emit ultrasonic calls were not retrieved. No further work on ultrasounds in rodents has been carried out until 1965, when Noirot's studies on maternal behaviour in mice led her to extend the observations of Zippelius and Schleidt [23]. In particular, Noirot showed that olfactory and auditory stimuli increased retrieving, licking and nest building responses in female mice when exposed for 5 min to a 1-2-day-old pup hidden in a perforated metal box [24]. A very clear demonstration that isolation calls do affect the searching behavior and probably also initiate the retrieving response of lactating females was given by Sewell [2] in a playback experiment. Lactating females of the species *Apodemus sylvaticus* entered more often to the compartment containing the loudspeaker emitting the relevant acoustic stimuli then to the compartment with the background noise or artificial stimuli, supporting the communicative value of pup vocalizations for the mothers of this species. Similar result have been obtained by Ehret and Haack [25] in playback experiments on *Mus musculus* lactating females (strain NMRI). They showed that females respond (in a two-alternative choice test) not only to natural calls but also to model calls consisting of bandpassed noise of variable bandwidth with noise energy in the frequency range of the natural calls (about 40-80 kHz) [25]. Starting from these pioneering studies, several other studies showed that USVs elicit approach and retrieval [26-29], and reduce attacks or rough manipulation by the dam [30,31]. Dam behavior modulates the number of ultrasonic calls uttered by the pups in social isolation conditions. Number of calls emitted by pups with intact hearing strongly decreased when these pups were cross fostered to deaf dams [32]. A relationship between maternal responsiveness and pup calling rate has been confirmed more recently in a study comparing C57BL/6 and BALB/c maternal responsiveness to USVs [33].

Following these behavioral studies, primarily devoted to study the functional role of USVs, several pharmacological studies have been carried out to evaluate the role of different neurotransmitter systems on the regulation of USV signaling in rodents. Nearly 30 years ago, various authors showed that pharmacological treatments clearly affect USV emission patterns [34-36]. Since then, effects exerted by several compounds on USVs has been extensively investigated [26,37-39]. Most of the pharmacological studies concern drug modulation of

***Corresponding author:** Maria Luisa Scattoni, Neurotoxicology and Neuroendocrinology Section, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Rome, Italy; Tel: +39-06-49903104; Fax: +39-06-4957821; **E-mail**: marialuisa.scattoni@iss.it

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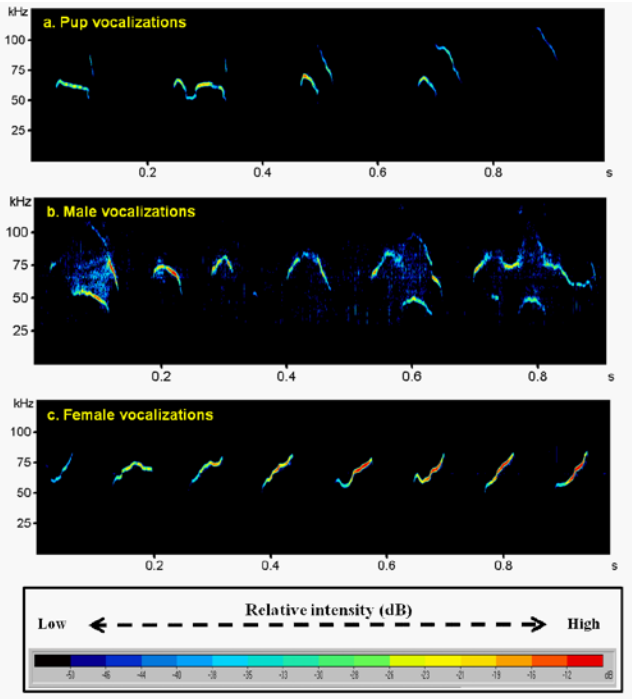

Figure 1: Spectrograms of call sequences emitted by mice at different ages and social contexts.

Panel a. Pup vocalizations emitted at PND 8 during the maternal separation paradigm.

Panel b. Courtship vocalizations emitted by a male when socially interacting with a receptive female.

Panel c. Ultrasonic vocalizations emitted by a resident female towards an intruder female.

Time (in seconds) is indicated by the X-axis, frequency in kHz is indicated by the Y-axis, and relative intensity or loudness is indicated by color (see colored bar at the bottom of the figure).

neonatal USV emission. Generally, pharmacological agents that act on GABA and/or 5-HT receptors and that alleviate anxiety in humans also reduce the emission of pup USVs [40-43]. Also glutamatergic drugs affect pup vocalizations [44].

Since these pharmacological studies clearly proved that USVs are affected by anxiolytic and anxiogenic drugs, researchers used ultrasonic vocalizations in mouse pups separated from their mothers as a test for emotional behavior early in postnatal life. Winslow and colleagues suggested that the amount of USVs in response to separation and isolation from the mother and littermates can be considered as a measure of primitive separation anxiety and that these calls can be predictive of adult emotionality [21].

More recently this extensive ethological/psychobiological and psychopharmacological knowledge of rodent ultrasonic vocalizations has been exploited in the study of behavioral phenotyping of genetically modified mouse lines, and in particular in those lines modeling neurodevelopmental disorders in which social communication deficits are one of the core symptoms, as Autism Spectrum Disorders (ASD).

Verbal and non verbal communication deficits are a cardinal feature of the autism spectrum disorders. In a child language task, autistic individuals assign stress to the wrong syllables of a word [45-47] and have difficulties modulating pitch and volume of their speech [47]. Some aspects of autistic speech seem to incorporate all three features of the diagnosis: repetitive behaviors, deficits in the ability to express emotions and deficits in communication. In fact, some children with autism repeat certain sounds, syllables, or words more than typically developing children [47] or fail to use appropriate patterns of intonation to communicate. Prosody can be monotonic, minimally pitched or energy modulated, or it can be amplified in pitch range or even singsong-like, masking dynamics in emotional status [48].

Since the field of mouse models of ASD has dramatically expanded in the last years, growing number of studies (Figure 2) have attempted to analyze, at different level of methodological complexity, USV patterns of ultrasonic vocalizations, and most of them report alterations in mutant mice and/or pups. In table 1, we report data analyzing USVs in ASD models. The following two paragraphs report an overview of results in the pup and adult vocalization tests.

Pup Ultrasonic Vocalizations

Pup isolation-induced ultrasonic vocalizations are whistle-like sounds with a single component at frequencies between 30 kHz and 90 kHz [49]. Calling rate always follows a strain-dependent ontogenetic profile, usually peaking between the fifth-eighth day after birth and then progressively decreasing till zero around the second postnatal week [50,51]. Any sort of deviation from this established ontogenetic profile can been considered an hallmark of altered neurodevelopment. However, not all studies analyzed number of calls at different time points throughout the first two postnatal weeks of age. Several authors rather analyzed USVs only at one single day (mostly PND 7-8) [20,21,52-60] giving for granted that this day corresponds to the peak of USV emission in their mouse strain. Unfortunately, strains differing in genetic background have different peaks of emission ranging from PND 3 in the C57BL/6J till PND 6-8 in the BTBR and FVB strains [17]. For this reason, researchers should either be aware of the ontogenetic profile of USV emission of the genetic background of their mutant lines and select the "right" peak day, or assess the entire USV profile throughout the first 12 days of postnatal life. This latter option definitively appears more informative [17,61-75].

Another aspect to consider when comparing data from different experiments is length of USV recording time. Usually pups vocalize for a brief period after separation from the mother, rapidly habituate, thus remaining silent after few minutes of social isolation. Most of the data available are from experiments with recording session lasting three [52,57,61-63,68,70,71,75], four [55] or five minutes [17,53,56,58-60,65,69,72-74]. Few papers reported a shorter isolation time than two

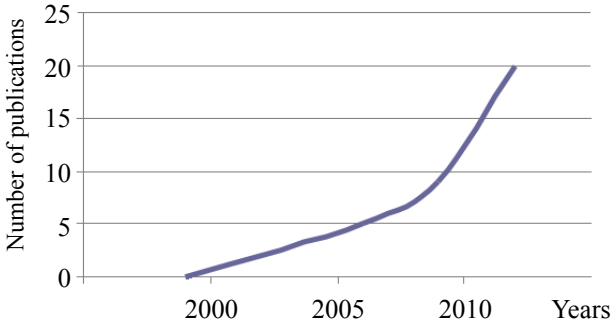

Figure 2: The rising number of publications about ultrasonic vocalizations in Autism animal models over the past 15 years. The graph has been plotted by searching the terms "ultrasonic vocalizations" and "autism animal models" on PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

Experimental test	PUPS		ADULTS				
Mouse models	Pup isolation	Maternal potentiation	Male - female Social interaction	Female - female Social interaction	Male - male Social interaction	Female urine exposure	References
Avpr1b + / - KO	=	↓		=			[72]
Avpr1b - / - KO	=	↓		↓			[72]
BTBR	↑		↓	↓	↓	↓	[17,87,99]
Cadm1 - / - KO	↓						[52]
Dlg4 - / - KO			↓				[89]
Dvl1 - / - KO	=						[67]
En2 + / - KO	=		=				[61]
En2 - / - KO		=		=			[61]
Exposure to chlorination by product	↓ (males)						[54]
Exposure to maternal immune activation	↓		↓		↓		[68]
Fmr1 - / - KO	=		↓; =				[56,95,96]
Foxp2 + / - KO	↓						[20]
Foxp2 - / - KO	↓						[20]
Foxp2 (R552H)/ - KI	↓						[53]
Foxp2 (R552H)/ (R552H) KI	↓						[53]
Mecp2 null	↑						[71]
Mecp2-308 -/ y	↓						[63]
Nlgn2 + / - KO	=						[60]
Nlgn2 - / - KO	↓						[60]
Nlgn3 - / - KO			↓				[91]
Nlgn3 (R451C) / (R451C) KI	↓ (males)						[62]
Nlgn4- / - KO			↓				[90]
Nr1neo - / - KO			↓				[94]
Orpm - / - KO	↓	↓					[69]
Oxt - / - KO	↓						[21]
Oxtr - / - KO	↓						[57]
Reln - / - KO (males)	↓(in handled)						[55]
Shank1 + / - KO	=					=	[59]
Shank1 - / - KO	↓					=	[59]
Shank2 + / - KO	=		=	=	=		[73]
Shank2 - / - KO	↑ (females)		=;↓	↓	=		[73,92]
Shank3 + / - KO	=		=; ↓			=	[75,93]
Shank3 - / - KO	=		=			=	[75]
Shank3 (e4-9) - / - KO				↓	↑		[88]
Slc 6A4 (56A)/ (56A) KI	↓						[58]
Tsc1 + / - conditional KO (Purkynje cells)	↑						[74]
Tsc1 - / - conditional KO (Purkynje cells)	↑						[74]
Tsc2 + / - KO		=					[77]
X non coding region KI (MALTT)	↑				↓		[64]
15q11-13 (maternal deletion 1,6Mb)	↑						[65]
15q11-13 (paternal duplication 6,3 Mb)	↑				=		[70]
17p11.2 (duplication 2Mb)	↓						[66]

Table 1: Ultrasonic vocalizations data in autism animal models. ↑ indicates a significant increase and ↓ a significant decrease in number of vocalizations when the different mouse models (listed in the first column of the table) are compared to their respective controls; = indicates vocalization rate comparable to their controls.

minutes [21,57,64,66,67], few papers a longer one (six: [20] or even fifteen minutes [54]).

Another source of variability in USV emission can be body temperature of pups, a physical parameter which is known to deeply influence pup vocalization rates [76]. Only few studies provide these data [17,21,54,58-63,71,72,75], thus hampering direct USV rate comparisons between different experimental settings.

In most of these studies, mouse pups with genetic alterations relevant for autism showed decreased number of vocalizations when separated from mother and siblings [20,21,52-55,57-60,62,63,66,68,69]. Both decreased and increased USV rates have been correctly interpreted as a developmental alteration within the social/communication domain. Interestingly, in the case of BTBR pups, in which a detailed quantitative analysis has been also carried out, it became clear that the higher vocalization rate was associated with a more limited vocal repertoire [17].

When assessing development of vocal responsiveness it is always recommendable to check for potential confounders as general somatic growth rate and initial acquisition of motor coordination competences (full neurobehavioral assessment [17,20,59-61,71,73,75], limited number of reflexes [53,66] and body weight gain [17,20,21,56,59-64,68,71,72,75,77]). This is needed because a reduction of USV rate when also accompanied by a delay in growth, maturation and motor coordination cannot be a selective marker of social communication deficit, but rather one of the signs of a general developmental deficit

impacting the health status of the pup. By contrast, if the USV rate is the unique alteration detected, this indicates a more selective impairment in vocal competences.

Maternal potentiation

In rat pups, an experimental paradigm has been developed to increase USVs. It consists of two consecutive separations: a five minute separation, followed by five minutes of contact with the mother, followed immediately by a second five minute separation [78]. This procedure leading the increase in vocalizations, called "maternal potentiation", has been extensively characterized during the second postnatal week in the rat species [79]. A detailed analysis conducted in 10-day-old neonatal rats showed that maternal reunion after maternal separation not only increases the subsequent calling rate but also induces qualitative changes in ultrasonic emission, namely increased average amplitude and average bout size (i.e. number of USV/bout) [80]. Later data showed that maternal potentiation of USV is a robust phenomenon not species-specific for rats, since it also occurs in guinea pigs [81] and some mouse strains [16,77].

Other findings indicate that maternal potentiation of USVs is not as robust in mice, and is extremely strain-dependent [72,82]. Data collected in C57BL/6J 8-day-old pups indicate that maternal potentiation of USVs can be detected using an experimental protocol modified from the rat one, with reunion occurring in the home cage with both mother and littermates [16]. Data from a mouse line with a null mutation in the mu-opioid receptor confirmed maternal potentiation in 12-day-old wildtype controls (C57BL/6J background strain), not in mutant pups [69]. Similarly, maternal potentiation was detected in wildtype animals from a line of Avpr 1b receptor knockout mice with a mixed C57BL/6J and 129/SvJ genetic background: in 9-day-old wildtype pups, maternal potentiation was found both in terms of number and duration of calls, the latter appearing as more sensitive parameter than number of calls, but no evidence of increase in vocalization rate after maternal reunion was found in heterozygous and homozygous mutant pups [72].

The maternal potentiation paradigm, together with the exposure to unfamiliar adult male odor (which is known to inhibit USV emission in pups, an adaptive response, since unfamiliar males are potentially infanticide [83]), offers the possibility of modulate vocalization rates as a function of external stimuli. These paradigms can therefore be particularly suitable to behavioral phenotyping of ASD models [69,72].

Adult Vocalizations

Under selected experimental conditions, emission of USVs (ranging from 40 to 80 kHz, mean duration 80 ms) is a consistent and robust phenomenon also during adult social interactions and is considered an index of social interest and motivation [16,84,85]. In fact, both in the male-female and female–female social interaction tests, ultrasonic vocalizations have been positively correlated with social investigation, such as anogenital sniffing [8,84,86,87].

The analysis of adult mouse vocalizations has been extended to ASD mouse models. With exception of male Shank 3 mutants [88], in all the remaining mouse lines tested, vocalization rates were significantly lower in mutants when compared to wildtype littermates [64,68,72,73,87-92]. Interestingly, in most of the lines analyzed, only homozygous mutants vocalize less than wildtype controls, whereas heterozygous show vocalization rates comparable to wildtype ones [72,73,93].

Male-female

Since children with autism show a sex ratio of 4:1 (male to female), behavioral phenotyping of animal models has primarily focused on male mice. The male-female interaction is therefore the most popular test for detecting the communication deficit in mouse models of autism at adulthood [61,68,73,75,87,89-96]. This is because in this social context, males vocalize shortly after the encounter with the female in association with the anogenital sniffing [97,98].

In this test, it is possible to evaluate the first approach of the male to a sexually receptive female, the ongoing social investigation and the associated USV emission [97,98]. Despite the wide use of the male-female paradigm in laboratories dealing with behavioral phenotyping of mouse model of autism, there is not a unique procedure and several sources of variability must be taken into account.

Session length has not been standardized and can vary from three [68,73,91] to five [61,75,87,89,92-95], or ten minutes [90]. Variable lengths of the social interaction session can prevent meaningful direct comparisons of data from different laboratories if data are not presented as mean value per minute throughout the session but only as average total means (low values of last part of the session).

Another source of variability among different settings is certainly represented by previous experience with females of the male subjects tested. Indeed, repeated prior exposure to an unfamiliar female can maximize probabilities of male vocalizations [68,73]. Also the strain (or genotype) of the female partner in the male-female test can affect male behavioral responsiveness: the commonest choice is a wildtype female [61,73,75,89,92-94] or a female of the same genetic background of the tested mice [87,90,91,96], more rarely a totally unfamiliar mouse strain has been used [88,95].

A crucial aspect of this test is the assessment of the female sexual receptivity [61,68,73,75,87,89-94,96]. Indeed, when it is not specifically evaluated, data are definitively weaker (male variability may increase because of exposure to females in different phases of the estrus cycle and the number of non-vocalizing males can dramatically increase).

Exposure to female urine

Another experimental paradigm utilized in behavioral phenotyping of mouse models of autism is the exposure to female urine, in which male reactivity to the presence of olfactory cues from a sexually receptive female [59,75,99] is detected in terms of male vocalizations and urine scent marking across the experimental arena. So far few researchers have used this test [59,75,99] although it is easy to perform and does not include the management of female subjects. One limitation is that no additional measurement of social behavior can be concomitantly associated with the vocal response.

Female-female

Few studies have analyzed mouse female USVs. Female mice emit a large number of USVs, at absolute rates comparable to those of the male–female interaction. Ultrasonic vocalizations are emitted during the first minutes of social interaction only by the resident female, not by the intruder one [6,84,97] in concomitance with high levels of social investigation of the intruder. Recently, this paradigm has been applied also in animal models of autism [72,73,87,88].

Male-male

Males emit ultrasonic vocalizations exclusively during non aggressive encounters. Indeed, during resident-intruder tests, that are characterized by high levels of aggressive behavior, only audible vocalizations have been detected primarily in association with defensive

postures, and have been therefore considered as stress-associated vocalizations [6,85].

Ultrasonic vocalizations during male-male interactions are thus detectable only when subjects belong to mouse lines characterized by reduced levels of aggressive behaviors featuring low levels of social interactions [64,68,73,87,88]. In most of the mouse lines, significant deficits were evident in male mouse models of ASD, with the exception of Shank 2 knockout mice: in this data set, however, vocalization rates were so low (also in wildtype controls) that, as admitted by the authors, it was rather unlikely to detect significant reduction in mutants [73].

Analysis of the Vocal Repertoire

The development of digital sound spectrographic analysis, which occurred in the last 10 years due to progress in computer technology, has allowed collecting further information about genetic factors shaping the USV responses, and provided an additional and more detailed source of information about USV structure compared to analysis based only on vocalization rate, which has so far been the most utilized method [9,16,17]. This new technique has been applied to mouse model of autism [17], analyzing and classifying calls emitted by three different inbred strains of mouse pups (C57BL/6 J, 129X1 and FVB/NJ) and comparing them with the BTBR, an inbred strain that displays several behavioral traits relevant to autism. Ten categories of calls have been defined according to the internal frequency changes, duration and sonographic shape. Such analysis revealed that BTBR pups emitted a narrower repertoire of calls, an unusual pattern that resembles the atypical vocalizations seen in some autistic infants [100-102]. More recently, analyses have also been carried out in other mouse models [56,68,73].

Manual detection and categorization can be performed only by experienced personnel able to discriminate calls from background noise. This qualitative analysis is currently extremely time-consuming and different laboratories are now focusing on development of a automatic system to detect and categorize calls.

Conclusions

The analysis of USVs in neonatal and adult ASD mouse models provide different information because they underlay two well separated motivational domains (mother-infant attachment *vs* sexual preferences/courtship behavior). USVs resulting from these two different age-periods not always overlap (e.g. see BTBR USV profile of pups opposite to the adult one [17,87]).

On one side, the neonatal USV assessment is the only one allowing to identify social communication deficits in early phases of development in accordance with the onset of ASD pathology in humans [103]. It could be therefore preferred when designing experiments aimed at either characterization of early behavioral markers in ASD mouse models or of preclinical evaluations of innovative treatments in early stages of the pathology.

On the other, adult USV assessment in the male-female setting is the best choice if only adult assessment of social communication is feasible (e.g. in laboratories with expertise in adult mouse behavioral phenotyping and not in neonate one). Indeed, the alternative behavioral test to evaluate social communication in mice is the social transmission of food preferences, but this test has been originally developed in the rat species as a social "learning" test (for a review see [104]) and even if the establishment of preference for a novel food can be assessed with minimal delay from interaction with rat demonstrator, it still remains a social learning test. Recently, neurobiological basis for this test have been elegantly clarified in terms of selected response of carbon disulphide receptors [105], a biological mechanism associated with breathing function and not with social communication.

Future Directions

Whereas USV rates have been extensively characterized, classification of call categories, sequences of call categories, and prosody have to be expanded and further explored.

As the diagnostic criteria for ASD are being changed (now including only two core symptoms: i) Social/communication deficits and ii) Fixated interests and repetitive behaviors) the relevance of complete assessment of the USV profile in ASD mouse models will be progressively increased in the next years.

Playback of recorded vocalizations during social encounters, and scoring of socially appropriate responses to the calls, are the optimal experimental paradigms to deeply evaluate the role of ultrasonic vocalizations in rodent communication [11,106]. Further analyses using context-specific playback experiments will be useful to discover whether: i) mice actually "communicate" biologically significant information to each other using ultrasonic vocalizations; ii) different categories or different sequences code for different meanings. An accurate analysis of ultrasonic emissions could thus provide a reliable assay to evaluate normal and altered communication profiles in greater detail.

A single playback experiment has been carried out in a mouse model of autism: mu-opioid receptor knockout male mice display a reduced exploratory activation, when compared to wildtype controls, upon exposure to previously recorded female USVs [107]. This behavioral paradigm has not been used so far in other mouse models, probably because it requires an extensive experience with handling and recording USV files and dedicated equipment, but it can be foreseen that when adequately applied such paradigm could provide detailed and innovative information on different communicative aspects of USVs in mouse model of autism.

Other future directions worth to be pursued are the possible application of USV responsiveness and USV qualitative analyses as indices of the effect of different therapeutically interventions in preclinical studies using mouse models. A number of preclinical studies, even when identifying significant ameliorative effects in ASD mouse models in terms of social behavior, did not analyze vocalizations after treatment [108-120]. So far only a single study has analyzed and detected an increase in vocalization rate, namely a treatment with minocycline, a tetracycline antibiotic with reported effects also at CNS level in a mouse model of X-Fragile [96]. Hopefully, these data will pave the way for inclusion of USV assessment other as a routinary test in behavioral phenotyping of ASD models also in preclinical drug testing.

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