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SPECIAL ISSUE REVIEW

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Mouse and rat ultrasonic vocalizations in neuroscience and neuropharmacology: State of the art and future applications

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Abstract

Mice and rats emit ultrasonic vocalizations (USVs), which may express their arousal and emotional states, to communicate with each other. There is continued scientific effort to better understand the functions of USVs as a central element of the rodent behavioral repertoire. However, studying USVs is not only important because of their ethological relevance, but also because they are widely applied as a behavioral readout in various fields of biomedical research. In mice and rats, a large number of experimental models of brain disorders exist and studying the emission of USVs in these models can provide valuable information about the health status of the animals and the effectiveness of possible interventions, both environmental and pharmacological. This review (i) provides an updated overview of the contexts in which ultrasonic

Abbreviations: 6-OHDA, 6-hydroxydopamine; ASD, autism spectrum disorder; BTBR, black and tan brachyury; Cacna1c, calcium voltage-gated channel subunit alpha1 C; CB1-KO, cannabinoid receptor 1 knock-out; CB1r, cannabinoid type-1 receptor; CNN, convolutional neural network; Cntnap2, contactin associated protein-like 2; CPP, conditioned place preference; D1 and D2-like receptors, dopamine 1 and 2 like receptors; Db, decibel; DRT, dopamine replacement therapy; ECS, endocannabinoid system; FM, frequency modulated; Fmr1, fragile X mental retardation syndrome 1; FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; HIE, hypoxic ischemic encephalopathy; Hs, hours; IGF-2, insulin-like growth factor-2; kHz, kilohertz; KO, knock-out; L-DOPA, L-3,4-dihydroxyphenilalanine; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MIA, maternal immune activation; MLX, meloxicam; MP, multilayer perceptron; mPer1, mouse period1; ms, milliseconds; MUPET, mouse ultrasonic profile extraction; NAmb, nucleus ambiguus; NDDs, neurodevelopmental disorders; NF-κB, nuclear factor kappa B; Nlgn, neuroligins; NTS, nucleus tractus solitarius; P2X4R, purinergic P2X receptor 4; PAG, periaqueductal grey; PD, Parkinson's disease; PND, postnatal day; PTSD, post-traumatic stress disorder; RF, random forests; SVM, support vector machines; UBE3A, ubiquitin protein ligase E3A; USVs, ultrasonic vocalizations; WAAVES, WAV-file automated analysis of vocalizations environment specific; WT, wild-type.

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calling behaviour of mice and rats has particularly high translational value, and (ii) gives some examples of novel approaches and tools used for the analysis of USVs in mice and rats, combining qualitative and quantitative methods. The relevance of age and sex differences as well as the importance of longitudinal evaluations of calling and non-calling behaviour is also discussed. Finally, the importance of assessing the communicative impact of USVs in the receiver, that is, through playback studies, is highlighted.

KEYWORDS

animal models, neurological disorders, neuropharmacology, neuropsychiatric disorders, ultrasound playback

1 | INTRODUCTION

The emission of ultrasonic vocalizations (USVs) is a major component of the behavioral repertoire of several rodent species (Fernández-Vargas & Johnston, 2015; Hennessy et al., 2006; Ma et al., 2014; Rendon et al., 2015; Simola & Brudzynski, 2018a; Wöhr & Schwarting, 2013). USVs emitted by mice and rats have been extensively studied and characterized in terms of behavioral significance and nature of the contexts that elicit calling behaviour. Several lines of evidence indicate that USVs serve important communicative functions, for example, to establish social contacts with conspecifics (Brudzynski, 2018; Wöhr, 2018; Wöhr & Schwarting, 2013). Importantly, USVs may reflect the arousal and/or affective state of the animal. Changes in arousal and/or affective state, for example, triggered by contextual changes and/or other relevant internal or external stimuli, are believed to be associated with changes in the emission of USVs, that is, reflecting low versus high arousal and/or a positive versus negative affective state of the animal (Brudzynski, 2018, 2021; Ehret, 2018; Premoli, Memo, & Bonini, 2021; Simola & Granon, 2019; Wöhr, 2018; Wöhr & Schwarting, 2013). Therefore, the emission of USVs in mice and rats is increasingly being used as a behavioral marker of sociability and affectivity in the fields of neuroscience and neuropharmacology. Emission of USVs originates from specific anatomical and physiological mechanisms (see below) that may be altered in experimental rodent models of human disease, and such alterations may eventually result in the occurrence of qualitative and/or quantitative changes in calling behaviour. In fact, the tight link between USV emission and the expression of arousal and/or emotional states that exists both in mice and rats may help to reveal abnormalities in perceptual systems, arousal and/or affect regulation. Accordingly, measuring USV emission and the changes in calling behaviour of mice and rats may provide valuable information on the health status of the animals. It can thus be hypothesized that qualitative and quantitative alterations in distinct USV parameters can be linked to specific abnormalities in arousal/emotional states in several experimental mouse and rat models of human disease, as it will be reviewed extensively here. Hence, USV emission can be seen as a behavioral readout that allows to assess the effectiveness of environmental and/or pharmacological therapeutic interventions aiming at the correction of such alterations. For the preparation of this review, scientific articles published in peer-reviewed journals were taken into consideration. The various scientific papers were used to document the state of the art of the topic (USVs in neuroscience and neuropharmacology) and were critically commented to draw conclusions on the role of USVs as valuable tool with high relevance for preclinical research in neuroscience and neuropharmacology. Furthermore, we also reported how the study of rodents' ultrasonic vocal behaviour is evolving in the light of the new methods for the acquisition and analysis of USVs.

1.1 | Anatomical origins and physiological mechanisms of USV production in mice and rats

Several studies have identified the larynx as the source of USVs in rodents (Riede, 2011; Riede et al., 2017; Roberts, 1975a). Roberts (1975a, 1975b, 1975c) was one of the first researchers to study the rodent laryngeal anatomy and its relevance for USV production. His hypothesis, called *hole-tone whistle model*, posited that USVs are generated by a hole tone whistle mechanism in which two spaced circular orifices produce a whistle (Roberts, 1975b). Briefly, airstream leaves a circular opening formed by the glottis and travels through a second hole placed downstream from the glottis (Brudzynski & Fletcher, 2010; Riede, 2013; Roberts, 1975b, 1975c). In addition, other mechanisms

for USV emission were recently proposed such as the *planar impinging jet model* based on observations made in the excised laboratory mouse larynx. This model theorizes that the glottal airflow impinges perpendicularly on a planar surface formed by the thyroid cartilage or by parts of the epiglottis (Mahrt et al., 2016). Accordingly, USVs would be generated by feedback between downstream convecting coherent flow structures from the glottis and upstream-propagating acoustic waves. The upstream propagating acoustic waves are generated by impingement of the coherent flow structures on the planar inner laryngeal wall. Finally, Riede and colleagues (Riede et al., 2017) proposed another model called *edge-tone mechanism* that identifies the intralaryngeal air sac (ventral pouch) and the alar cartilage located at the ventral pouch entrance as key players in the emission of USVs. In this model, the airstream exiting the glottis is directed at the edge of the alar cartilage resulting in an undulating flow that interacts with the ventral pouch resonance functioning like a Helmholtz resonator. Therefore, sound frequency is determined by glottal airflow speed, ventral pouch dimensions and glottis-to-edge distance. This mechanism requests intrinsic control of laryngeal muscles and is essential for acoustic variations in mice and rats (Riede et al., 2017). Laryngeal muscles are innervated by the vagal motor nucleus ambiguus (nAmb); Kamitakahara and colleagues demonstrated that conditional embryonic deletion of the gene encoding the MET receptor tyrosine kinase in the developing brainstem results in a massive (about one-third) loss of these motor neurons in the nAmb, causing highly penetrant, severe deficits in the emission of USVs in early postnatal life that persist into adulthood, thus suggesting a critical role for MET receptor in the regulation of USV emission (Kamitakahara et al., 2021).

In mice, rats and other rodents, the larynx is responsible for swallowing, respiration and vocal production. Neural control of these functions and laryngeal morphology may vary during development and this could explain the occurrence of changes in the acoustic properties of USVs throughout an individual rodent's lifespan (Fernández-Vargas et al., 2022). However, the anatomical elements of the larynx that determine the emission and acoustic parameters of USVs in mice and rats have not been unambiguously identified yet. Ultrasonic whistle emission seems to depend on the presence of the ventral pouch and alar cartilage (Riede et al., 2017). Indeed, the presence of damages in the ventral pouch and/or alar cartilage compromises the capability of rats to produce USVs (Riede et al., 2017). Moreover, the spectral content of calls is defined by laryngeal anatomy (Riede, 2018). Therefore, the existence of differences in larynx morphology can explain differences in the acoustic features of USVs across different experimental models of disease in mice. For example, lower values of sound amplitude and sound frequency were found in case of USVs emitted by mice deficient for the clock gene *Period1* (*mPer1*$^{-/-}$ mice) in comparison with wild-type (WT) mice, and these abnormalities were linked to alterations in laryngeal morphology. Indeed, according to Mahrt's theory, the altered laryngeal morphology observed in these mutant mice could lead to variations in the impingement length of airstream and determine the difference in sound frequency compared to those of WT mice. In addition, based on *edge-tone theory*, the large larynx found in *mPer1*$^{-/-}$ mice might concur to determine the peculiar acoustic features of the USVs emitted by this strain, since the sound frequency of USVs is influenced by the size of laryngeal ventral pouch (Bahlmann et al., 2019). Also, other authors who studied acoustic variations of USVs caused by morphological differences in the larynx in mouse strains described lower mean fundamental frequency and higher amplitude of infant calls as well as longer duration and higher rate of calls in DBA/2J mice compared to C57BL/6J mice (Ashbrook et al., 2018). The fundamental frequency differences between these two mouse strains have been explained based on differences in laryngeal size, laryngeal control, and/or in breathing movements (Ashbrook et al., 2018). In pups, breathing and its control are very important for regulating USV emission (Hernandez-Miranda et al., 2017). Therefore, higher duration and number of USVs emitted by pups of the DBA/2J strain could be explained by an impairment in breathing movement control (Ashbrook et al., 2018). Laryngeal dysfunction can as well explain the presence of altered USV emission in rats, as demonstrated by studies that observed qualitative changes in the acoustic parameters of 50-kHz USVs emitted in rat models of dopamine hypofunction and aging (Johnson et al., 2015). In addition, studies in rats have demonstrated that lung pressure controls sound amplitude and that an increase in lung pressure is linked to an increase in USV sound intensity (Riede, 2011). Future studies focusing on the relationship between lung pressure, respiratory parameters and acoustic features of USVs are necessary to better understand the involvement of breathing control in their emission, both in mice and rats.

Finally, recent findings suggest that the emission of USVs is a complex phenomenon and requires the control of phonation and respiration, as well as neural control of laryngeal function that involves forebrain structures and brainstem motor neurons (Jürgens, 2002, 2009; Simonyan, 2014). The midbrain periaqueductal grey (PAG) plays a relevant gating function in this respect.

Indeed, damages to the PAG provoke mutism (Esposito et al., 1999; Jürgens, 1994, 2002, 2009), whereas activation of PAG can elicit USVs in several animal species (Bandler & Carrive, 1988; Kyuhou & Gemba, 1998; Lu & Jürgens, 1993; Schuller & Radtke-Schuller, 1990; Zhang et al., 1994). Tschida and colleagues (Tschida et al., 2019) found a specific subset of PAG neurons that are active during courtship USV production in male mice. Interestingly, these USVs-related PAG neurons project to downstream vocal-respiratory centres that are important for temporal and spectral features of vocalizations (Tschida et al., 2019). Moreover, genetic silencing of USV-related PAG neurons blocks USV production, whereas their activation elicits calling behaviour even in the absence of female cues (Tschida et al., 2019). The PAG receives input from several forebrain structures that are involved in high-order brain functions, such as the regulation of social behaviour and emotions (Newman, 1999; O'Connell & Hofmann, 2011). In addition, the PAG projects to brainstem motor neurons playing an important role in the activation of the larynx and respiratory organs (Fernández-Vargas et al., 2022). Another important area that is involved in ultrasonic calling behaviour is the nucleus tractus solitarius (NTS), located in the hindbrain. This 'vocal' nucleus is essential for USV emission, as demonstrated by the fact that genetically-modified mice lacking the NTS are mute and unable to produce the expiratory pressure required for USV emission (Hernandez-Miranda et al., 2017). Furthermore, the NTS contains premotor neurons that project to spinal (L1) and nAmb motor neurons that control expiratory pressure and laryngeal tension (Hernandez-Miranda et al., 2017).

1.2 | Mouse ultrasonic vocalizations: Acoustic parameters and contexts eliciting calling behaviour

Mice emit vocalizations both in the audible and ultrasonic frequencies (Ehret & Bernecker, 1986; Zippelius & Schleidt, 1956). In mice, USVs are characterized by sound frequency ranging from 30 to 110 kHz (Holy & Guo, 2005) and their use represents a major means of communication in both young and adult animals. USVs can be analysed both from a quantitative and a qualitative point of view; in fact, it is possible to study the number of calls uttered and their duration, but also their spectrographic characteristics (e.g., peak frequency, peak amplitude, bandwidth, etc..). Based on a combination of duration and spectrographic characteristics (e.g., presence of frequency steps) USVs have been classified into different types, such as 'short', 'chevron', 'two-steps' calls, although the precise terminology may vary across studies (Gaub et al., 2016; Grimsley et al., 2016; Holy & Guo, 2005; Scattoni, Gandhy, et al., 2008). The 'meaning', or rather the potential emotional valence ('positive' versus 'negative') of the different types of calls is not yet known in mice (in contrast to rats, as described in detail below). Hence, several ongoing studies attempt to attribute a precise communicative meaning to different call types; unravelling the potential emotional 'meaning' of USVs would indeed have crucial repercussions for neuroscience research on mouse models, especially in the clinical/pharmacological field.

Mouse ultrasonic communication is intrinsically linked to social behaviour, and the study of USVs has become a valid assay in the behavioral phenotyping of mouse models, especially in the context of social dysfunction and related pathologies. Mice emit USVs in specific and well-defined situations: as pups when separated from the mother; as juveniles and as adults during reciprocal social interaction with same-age peers.

In pups, USVs present sound frequency ranging from 30 to 90 kHz, duration of 10–200 ms and sound pressure levels of 60–100 dB; almost all types of calls detectable in adults are already present (Bell et al., 1972; Branchi et al., 1998; Grimsley et al., 2011). USV production follows a clear strain-dependent ontogenetic profile from birth to postnatal day (PND) 14–15, which is usually characterized by an increase in call emission in the first 5–6 days post birth, followed by a rapid decrease leading to their disappearance in the second week of life (Branchi et al., 2001; Elwood & Keeling, 1982). Pups emit USVs to induce retrieval from the mother, as in the first postnatal days (PNDs) they are totally dependent upon her, because of their sensorial immaturity and inability to thermoregulate themselves. These 'whistles of loneliness' ('Pfeifen des Verlassenseins'; Zippelius & Schleidt, 1956) represent the first examples of communication between the mother and its offspring (D'Amato et al., 2005; Hernandez-Miranda et al., 2017) aimed at inducing a caregiving behaviour in dams (Smotherman et al., 1974). Furthermore, variations in the spectral content, both frequency and amplitude, of USVs may mirror the emotional states of pups (D'Amato et al., 2005; Lahvis et al., 2011). Indeed, the presence of a threatening stimulus, such as the odour of an unfamiliar, potentially infanticidal, adult male, induces changes in the types of calls emitted by pups (Branchi et al., 1998). Furthermore, abnormalities in the development of emotional behaviour associated with the deletion of genes linked to social bonding and attachment behaviour, such as oxytocin or mu-opioid receptor genes, cause alteration in USV emission, with a significant reduction in maternal separation-induced USVs in pups (Moles et al., 2004; Winslow et al., 2000).

In juvenile mice, several characteristics of USVs are correlated with social behaviour and present a strain-dependent variability (Panskepp et al., 2007; Peleh et al., 2019). The production of USVs during adolescence is mostly triggered by social interactions between mixed-sex mice (Panskepp et al., 2007). In mice, the number of USVs gradually decreases and their duration becomes shorter from infancy to adolescence. This probably reflects their vital role during the early postnatal phase and their reduced relevance during social or territorial interactions. Interestingly, the mean frequency of USVs in juvenile mice is higher than in pups, suggesting a relationship between low-frequency calls and highly stressful situations (Peleh et al., 2019). Interestingly, developmental changes in acoustic parameters, such as increased sound frequency of calls emitted by juveniles compared to those emitted by pups, may be linked to more efficient motor coordination of vocal organs involved in USV production. Indeed, trachea and larynx grow and also the control of laryngeal muscles improves with the age of animals. Therefore, the sound frequencies produced by this mechanism may change, as reported in rat and mouse studies (Blumberg et al., 2000; Liu et al., 2003).

Compared to younger mice, USVs emitted in adulthood are substantially lower in frequency and shorter in duration (Grimsley et al., 2011). Both female and male mice emit vocalizations in adulthood, yet the context that induces a particularly large number of USVs in females is dyadic interaction with a female intruder. During this type of social interaction, USVs are used to establish affiliative-like social bonds (Moles et al., 2007; Moles & D'Amato, 2000; Zala et al., 2017). Nonetheless, the best characterized USVs in adult mice are those emitted by males to attract a female during courtship; these calls have a fundamental role for sexual behaviour and mating (Egnor & Seagraves, 2016). In a mixed-sex dyadic interaction, usually the male mouse emits USVs (Egnor & Seagraves, 2016; Hammerschmidt et al., 2012; Sugimoto et al., 2011). During a male–male social interaction instead, very few USVs are produced, and they serve to mediate competition over social status (D'Amato, 1991; Nyby et al., 1976; Zala et al., 2017) and to establish territorial dominance (Matsumoto & Okanoya, 2018). Both male and female mice emit USVs at a higher rate and higher sound frequencies during opposite-sex compared to same-sex interactions; furthermore, call emission is modulated based on the sex of a potential receiver, since male mice emit USVs with higher amplitude in presence of a male mouse compared to a female mouse (Zala et al., 2017). Experiments on C57BL/6 J mice, in a resident-intruder context, demonstrated that USVs emitted during male–female versus female–female social interaction are qualitatively different, with females producing longer and more complex calls compared to male mice; these sex-dependent qualitative differences of USVs were stable across multiple social encounters (Premoli et al., 2022). Beside the social context, other stimuli that induce the emission of USVs, albeit to a much lesser extent, include the exposure to a novel environment or to restrain stress, that is, situations both associated with anxiety-like behaviour and emission of low-frequency USVs (≤60 kHz) (Chabout et al., 2012; Lefebvre et al., 2020). In all these cases, mice produce USVs in response to a change in their environment. In addition, the increase or decrease in USV number is linked to the intensity of environmental change and this is explained by the level of arousal of the mouse (Ehret, 2018; Gaub et al., 2016). This idea has been hypothesized by Bell (Bell, 1974) and supported by subsequent studies that found a high level of arousal correlated with a high USV number especially during sniffing in male–female interactions (Gaub et al., 2016; Hammerschmidt et al., 2012; Scattoni et al., 2011; Zampieri et al., 2014). In particular, temporal features of USVs, such as call duration and inter-call intervals, reflect the state of arousal in mice, while intensity and spectrotemporal call complexity (overtones/harmonics and frequency jumps) can be indices of positive emotion levels (Gaub et al., 2016). Therefore, changes in specific acoustic parameters of ultrasonic calls appear to offer insights into affective states of mice.

1.3 | Rat ultrasonic vocalizations: Behavioral significance, acoustic parameters and contexts eliciting calling behaviour

Rats are social animals, and their behavioral repertoire is characterized by a rich set of social activities that includes the emission of USVs (Brudzynski, 2013; Wöhr & Schwarting, 2013). Three families of USVs have been characterized in rats that possess different acoustic parameters and behavioral significance. Infant rats emit the so-called 40-kHz USVs, which have a maximum sound frequency contained between 30 and 70 kHz. Rat pups generally emit 40-kHz USVs in response to isolation from the mother and nest; nevertheless, other situations, for example handling by humans or social contacts with the mother, can as well stimulate the emission of 40-kHz USVs (Schwarting & Wöhr, 2018; Wöhr et al., 2008). The emission of 40-kHz USVs is considered a behavioral marker of negative affect, and it is speculated that variations in the numbers of 40-kHz USVs emitted and/or in the structure of individual calls may indicate the

presence, and possibly the magnitude, of distress in infant rats (Iijima & Chaki, 2005). USVs emitted by young and adult rats are distinguished in the two families of low-frequency 22-kHz calls, which occur in aversive situations, such as predator exposure (Blanchard et al., 1991) or fighting (Vivian & Miczek, 1993), and high-frequency 50-kHz calls that occur in appetitive situations, most notably during rough-and-tumble play (Knutson et al., 1998) or when rough-and-tumble play is mimicked by means of the procedure of 'tickling' performed by an experienced human experimenter who is familiar to the rats (Panksepp & Burgdorf, 2000). Because of this link to tickling, the term 'rat laughter' is repeatedly used (Panksepp, 2005). High rates of 50-kHz USVs are further observed in response to drugs of abuse, most notably psychostimulants with a strong dopaminergic component, such as amphetamine (Wöhr, 2022).

Calls of the 22-kHz USV family have a maximum sound frequency that is typically comprised between 20 and 33 kHz, do not show significant modulation in their sound frequency and have a long duration (usually more than 300 ms) (Brudzynski & Ociepa, 1992). Nevertheless, 22-kHz USVs of short duration (i.e., lasting less than 300 ms) have also been identified (Barker et al., 2010; Brudzynski, 1994), although the behavioral significance of the latter calls is still to be defined. The emission of 22-kHz USVs marks the presence of arousal associated with stimuli that have negative affective valence and of an aversive emotional state, as corroborated by the evidence that rats utter 22-kHz USVs in situations that may be harmful to an individual animal and/or to a colony. These situations may either have social valence, for example, encounters with aggressive conspecifics or unfamiliar humans (Brudzynski & Ociepa, 1992; Panksepp et al., 2004), or be devoid of social valence, for example, the exposure to air puffs or the withdrawal from drugs that possess rewarding properties (Brudzynski & Holland, 2005; Oliveira & Barros, 2006). Moreover, the expectation of stimuli that have negative emotional valence may as well elicit the emission of 22-kHz USVs in rats (Kassai & Gyertyán, 2018). In consideration of the large variety of aversive situations that stimulate the emission of 22-kHz USVs, these calls can be regarded as a reliable behavioral marker of negative arousal and aversive emotional state in rats, consistent with the hypothesis that the emission of 22-kHz USVs could be an evolutionary equivalent of adult human crying (Brudzynski, 2019).

Calls of the 50-kHz USV family have a maximum sound frequency that generally ranges between 35 and 80 kHz, although calls with a maximum sound frequency higher than 80 kHz have been also included in this family (Brudzynski, 2013). Moreover, 50-kHz USVs have short duration (i.e., they usually last less than 30–40 ms) (Simola & Brudzynski, 2018b). Differently from 22-kHz USVs, 50-kHz USVs may display a rich modulation of their sound frequency. This has led to the further categorization of 50-kHz USVs in 'flat' calls, which display a nearly constant sound frequency within individual vocalizations, and 'frequency modulated' (FM) calls, which show substantial changes in sound frequency within individual vocalizations (Burgdorf et al., 2008; Wöhr et al., 2008). Previous studies have suggested that flat and FM 50-kHz USVs may have different functions. Thus, flat calls seem to serve mainly as a means of social coordination (Burgdorf et al., 2008; Wöhr et al., 2008). Conversely, FM calls appear to be the category of 50-kHz USVs more closely related to the presence of arousal associated with stimuli that have positive affective valence and of a positive emotional state in the emitting rat (Burgdorf et al., 2008, 2011). Besides, FM 50-kHz USVs have been further categorized in 13 subtypes based on their shape in the spectrogram, that is, features associated with the modulation in sound frequency (Wright et al., 2010). Nevertheless, limited information is currently available on the behavioral significance of the different subtypes of FM 50-kHz USVs (Burke et al., 2017; Simola & Costa, 2018). The hypothesis that rat 50-kHz USVs mark the arousal associated with stimuli that possess positive affective valence and the presence of a positive emotional state is corroborated by the evidence that these USVs are emitted in response to and/or in expectation of stimuli that may be beneficial to an individual and/or its colony. The situations that trigger the emission of 50-kHz USVs may have social valence, for example, non-aggressive contacts with either conspecifics or familiar humans (Bialy et al., 2000; Burgdorf & Panksepp, 2001) but may also be devoid of social valence, for example, the administration of drugs with rewarding properties or the performance of behavioral tests that evaluate emotionality or memory (Burgdorf et al., 2001; Costa et al., 2021; Ma et al., 2010; Rao & Sadananda, 2015; Reyes et al., 2021; Simola et al., 2010, 2012). Rats may emit 50-kHz USVs also in anticipation of appetitive stimuli of various nature, a response that is thought to mark the expectation of positive emotional states (Brudzynski & Pniak, 2002). Since a wide array of situations that have positive emotional valence can stimulate and/or modulate the emission of 50-kHz USVs, these calls can be considered a solid behavioral marker of positive arousal and positive emotional state in rats, in agreement with the hypothesis that the emission of 50-kHz USVs could be an evolutionary equivalent of adult human joy (Panksepp & Burgdorf, 2003).

2 | ULTRASONIC VOCALIZATIONS AS BEHAVIORAL MARKERS IN PRECLINICAL STUDIES ON AFFECTIVITY/SOCIABILITY IN MOUSE AND RAT MODELS OF BRAIN DISORDERS

There is multiple evidence demonstrating that USVs reflect both arousal and emotional states in social and non-social contexts. This has attracted attention on mouse and rat USVs as a straightforward behavioral marker for preclinical research on affectivity, perception of stimuli and social behaviour.

2.1 | Ultrasonic vocalizations in mouse experimental models of brain disorders

In recent times, mouse USVs have acquired increasing importance in the preclinical study of psychiatric and neurological disorders, starting from those characterized by deficits in communication and social interaction, such as neurodevelopmental disorders (NDDs) in general and autism spectrum disorder (ASD) in particular (Fischer & Hammerschmidt, 2011; Moy & Nadler, 2008; Scattoni et al., 2009; Simola & Granon, 2019). USVs have already been studied and characterized in several ASD murine models, including genetic and environmental origins. The USV differences that emerged in these models were both quantitative and qualitative; that is, they affected the number, mean duration, peak amplitude and frequency, as well as the types of calls.

For instance, *Fmr1* knock-out mice are used to study fragile X syndrome, a NDD, which implies intellectual disabilities, but represents also the most common monogenic cause of ASD (Pieretti et al., 1991; Rogers et al., 2001); *Fmr1* mutants present several alterations in ultrasonic communication, as it will be described in detail below.

The *BTBR* mouse strain is used as idiopathic model of ASD because it displays core symptoms typical of ASD patients, such as social deficits, impaired communication and repetitive stereotype behaviors, as well as neuroanatomical abnormalities (Faraji et al., 2018; Meyza & Blanchard, 2017). *BTBR* pups present increased call number, duration, peak amplitude, and decreased peak frequency on PND 8, and increased 'harmonics', 'two-syllable' and 'composite' calls compared to WT pups (Scattoni, Gandhy, et al., 2008). Both at adolescence and adulthood, they emit a reduced number of calls and with different qualitative repertoire compared to WT mice on the basis of different social interaction tests (Scattoni et al., 2011; Scattoni et al., 2013; Wöhr, Roullet, & Crawley, 2011; Yang et al., 2013).

There are several monogenic mouse models of ASD that present mutations/deletions in genes that regulate synaptic plasticity, a function that is also altered in patients with ASD. This includes contactin associated protein-like 2 (*Cntnap2*) knock-out (KO) mice, which present a mutation in a gene coding for synaptic cell adhesion molecule. They emit less USVs both as pups, on PND 3, 6 and 9, and as adults compared to wild-type (Brunner et al., 2015; Peñagarikano et al., 2015). Again, the neuroligin (*Nlgn*) mutant mice, *Nlgn2* KO pups, present reduced number of calls and total calling time on PND 7 compared to their WT littermates (Wöhr et al., 2013). Instead, *Nlgn4* KO mice emit calls with reduced duration and increased latency to first call compared to WT animals during adolescence (Ju et al., 2014), and reduced number of USVs also at adulthood (Jamain et al., 2008). Finally, USVs are altered in *Shank* KO mice (Monteiro & Feng, 2017): *Shank1* KO pups emit less USVs with increased peak frequency compared to WT littermates; moreover, a different clustering of USVs, with two main groups of calls (50–80 kHz and 80–100 kHz) (Sungur et al., 2016; Wöhr, 2014; Wöhr, Roullet, Hung, et al., 2011), was found on PND 8 in *Shank1* KO compared to WT pups. *Shank2* KO female pups emit an increased number of calls on PND 4 and 10 (Schmeisser et al., 2012), decreased number on PND 6 (Ey et al., 2013) and also a decreased call number at adulthood in a female–female social interaction test (Ey et al., 2013; Schmeisser et al., 2012). Furthermore, *Shank3* KO adult mice emit fewer USVs during the female–female social interaction test, and their emission is characterized by reduced duration and frequency modulation; on the contrary, in the context of male–female social interaction test, they produce more USVs, but shorter and with reduced frequency modulation compared to WT animals (Wang et al., 2011).

All these variations in USVs reflect communication impairments that can have a great translational value in preclinical research for the study of possible pharmacological and non-pharmacological interventions. For instance, minocycline, a tetracycline antibiotic, has been demonstrated to rescue the communication deficits of Fmr1-KO young mice (Rotschafer et al., 2012). Also, anxiolytics are able to modulate USV emission in pups (Fish et al., 2004). USVs can be also modulated by non-pharmacological interventions, for example, social enrichment: a maternal social enrichment on *Fmr1*-KO pups resulted in a weaker emotional response to maternal separation, that is, a reduced number of USVs in the pups of this mouse line (Oddi et al., 2015).

There are several data supporting the fact that an altered emission of USVs is accompanied by sensory and/or motor deficits in mouse models of pathology. In particular, 16p11.2 deletion syndrome mice display sensory and motor abnormalities, including higher nociceptive thresholds, a complete absence of acoustic startle responses, and hearing loss (Yang et al., 2015). Instead, *Nlgn2* KO mice present normal acoustic, tactile, and olfactory sensory information processing as well as sensorimotor gating, but deficit in motor performance (e.g., reduced exploratory activity and impaired rotarod performance) (Wöhr et al., 2013). *Fmr1*-KO mice, that present an ASD-like behavioral phenotype with social and communication deficits, also display sensory-motor alterations, such as hyperactivity, altered emotionality, sensory hyper-responsiveness (Gaudissard et al., 2017). Purinergic P2X receptor 4 (*P2X4R*) KO mice display marked alterations in sensory perceptions (e.g., deficits in acoustic startle reflex amplitude, significant sensorimotor gating impairments, and enhanced tactile sensitivity) and they showed significant reductions in social interaction and maternal separation-induced USVs in pups (Wyatt et al., 2013). Also, *Kirrel3* KO mice exhibit ASD-like behaviors, including social and communicative deficits, repetitive behaviors and sensory abnormalities (Hisaoka et al., 2018). All these data suggest a strict correlation between socio-communicative and sensorimotor impairments, suggesting a complex relationship between perceptual/motor system deficits and alterations in USV emission in mice.

USVs are also studied in non-genetic murine models, for instance, those of cerebral ischemia, as a behavioral marker of post-traumatic recovery; indeed, speech impairments are often negative consequences of an ischemic event. Using the transient middle cerebral artery occlusion (MCAO) murine model, Doran et al. (2015) demonstrated that injury to the left hemisphere reduced early vocalizations more profoundly than those to the right hemisphere. Also, neonatal hypoxic ischemia encephalopathy (HIE) leads to major deficits in language development; hypoxic ischemia induced by right common carotid artery transient ligation at PND 10 provokes a significant reduction in USV emission (Doran et al., 2019). Similarly, maternal post-traumatic stress disorder (PTSD) following trauma exposure during pregnancy in mice causes an increase in the number and duration of the offspring USVs at PND 9 (Golub et al., 2016). Finally, a toxicological study demonstrated that dioxin exposure during pregnancy caused decreased number of calls and their duration, with a reduction of complicated call types (i.e., chevron and wave) compared to controls (Kimura & Tohyama, 2018).

2.2 | Rat ultrasonic vocalizations in experimental models of brain disorders

Most of the investigations that have evaluated the emission of USVs in rat models of brain disorders have focused on neurodevelopmental and psychiatric disorders and have employed USVs as a behavioral marker to investigate the presence of alterations in stimulus perception and processing, emotionality and sociability. Nevertheless, some studies exist that have evaluated calling behaviour of rats in experimental models of neurological disorders, to reveal the presence of alterations in the laryngeal mechanisms that sustain the emission of USVs and reproduce at the preclinical level the changes in phonation that may occur in patients with neurological disorders. Overall, these investigations have demonstrated that measuring the emission of each of the families of USVs that have been identified in rats (i.e., 40-kHz USVs of infant rats, 22-kHz USVs and 50-kHz USVs of young-adult rats) can provide relevant information on how brain dysfunction alters affectivity, sociability and USV production (Krasko et al., 2021; Wöhr & Schwarting, 2013). Moreover, measuring USV emission in rat models of brain disorders may be a relevant experimental approach in the development of novel pharmacological agents to treat altered affectivity and sociability (Simola, 2015).

Most studies that have evaluated the emission of 40-kHz USVs in rat pups subjected to maternal separation used this behavioral response as a marker of affectivity and sociability. Some earlier studies have suggested that the emission of 40-kHz USVs in rat pups stems from physiological mechanisms that are not related to changes in the emotional state (Blumberg & Alberts, 1991; Blumberg & Sokoloff, 2001). However, this hypothesis is no longer tenable, and the available evidence indicates that the emission of 40-kHz USVs provides valuable information on early modifications in affective and social behaviour in rat pups (Shair, 2018). Accordingly, measuring the emission of isolation-induced 40-kHz USVs in rat pups is now a widely used approach to investigate how genetic and environmental factors, and the interaction among these factors, elicit negative affectivity at the early phases of life (Shair, 2018). Moreover, measuring the emission of isolation-induced 40-kHz USVs in rat pups may predict the manifestation in later life of behavioral phenotypes that are reminiscent of those observed in major neurodevelopmental and psychiatric disorders, such as anxiety, ASD, depression, or schizophrenia (Brunelli et al., 2015; Kirsten et al., 2015; Zimmerberg et al., 2005).

Similar to 40-kHz USVs in infant rats, the emission of 22-kHz USVs in young/adult rats may reveal the

presence of arousal associated with stimuli that have negative affective valence and the induction of a negative emotional state by aversive situations (Drugan & Stafford, 2018; Faraji et al., 2016; Jelen et al., 2003; Kassai & Gyertyán, 2018). Accordingly, 22-kHz USVs are nowadays a useful behavioral readout in studies of anxiety, since they are reliably elicited by stimuli that induce anxiety-like states in rats; moreover, measuring the emission of 22-kHz USVs can have predictive validity in the investigation of new anxiolytic agents (Sánchez, 2003). The emission of 22-kHz USVs has been used as a behavioral marker of negative affectivity also in rat models of neuropsychiatric disorders other than anxiety, like depression, drug dependence and schizophrenia (Barker et al., 2010; Barros & Miczek, 1996; Reno et al., 2017; Tunstall et al., 2009). Remarkably, the emission of 22-kHz USVs in rat models of neuropsychiatric disorders may reveal the presence of modifications in arousal and emotional states that are not, or are only partially, disclosed by traditional behavioral readouts, such as freezing behaviour or prepulse inhibition of startle reflex (Tunstall et al., 2009; Wöhr et al., 2005). Additionally, measuring the emission of 22-kHz USVs may be a relevant approach in studies on the interplay between exposure to aversive stimuli in early life and manifestation of negative affectivity in later life (Riaz et al., 2015; Swiergiel et al., 2007). Furthermore, modifications in the emission of 22-kHz USVs may anticipate the manifestation of pathological behavioral phenotypes, as in the case of alcohol consumption. For example, the occurrence of changes in the acoustic parameters of spontaneously emitted 22-kHz USVs can predict drinking vulnerability in various rat strains (Mittal et al., 2018; Reno et al., 2017).

In contrast to 40-kHz USVs and 22-kHz USVs, the emission of 50-kHz calls is widely interpreted as a marker of arousal associated with stimuli that have positive affective valence and of positive emotional states, thus revealing the presence of blunted or heightened affectivity. A reduced emission of 50-kHz USVs in response to appetitive homo- and heterospecific (i.e., tickling by a familiar experimenter) social stimuli has been reported in pharmacological rat models of psychotic-like states induced by phencyclidine or ketamine, two drugs that can produce a state of social withdrawal in experimental rodents (Boulay et al., 2013; Nikiforuk et al., 2013; Swalve et al., 2016). Similar findings have been obtained in rat models of NDDs, like the gestational exposure to proinflammatory agents or the social isolation at weaning (Kirsten et al., 2018; Tomazini et al., 2006), as well as in rat lines that are considered to model certain NDDs of humans (Burgdorf et al., 2013; Kisko et al., 2018). Taken together, these findings may suggest that a reduced emission of 50-kHz USVs in response to appetitive social stimuli may reflect the presence of blunted affectivity in rat models of psychosis and of NDDs. Regarding the possible use of 50-kHz USVs as a behavioral marker of heightened affectivity in rat models of neuropsychiatric disorders, recent studies have suggested that measuring the emission of these USVs may be of relevance to reveal the mood elevations that feature mania (Wöhr, 2022). Indeed, an increased emission of 50-kHz USVs has been demonstrated in pharmacological and non-pharmacological rat models of mania (Wendler et al., 2016; Wendler et al., 2019), and such a heightened calling behaviour has been proposed to reproduce types of manic episodes different from those that are reproduced by measuring conventional behavioral readouts, such as hyperactivity (Wendler et al., 2016). Furthermore, the emission of 50-kHz USVs has been extensively evaluated in rat models of drug dependence, to clarify how the positive emotional and motivational effects of drugs may influence the onset and maintenance of addictive behaviors. The latter studies have demonstrated that rats treated with drugs that possess rewarding properties and have abuse potential may emit 50-kHz USVs immediately after drug administration, a response that is thought to reflect an acute positive effect of drugs on the emotional state (Burgdorf et al., 2001; Simola et al., 2010). Moreover, rats treated with drugs that possess rewarding properties and have abuse potential may emit 50-kHz USVs when exposed, in drug-free conditions, to environmental cues previously paired with drug administration, a response that may reflect the presence of increased arousal stimulated by drug-associated stimuli and the occurrence of environmental conditioning to the affective properties of drugs (Costa et al., 2020; Ma et al., 2010; Simola et al., 2014, 2016). At the same time, it has been demonstrated that the emission of 50-kHz USVs may not fully overlap with other behaviors that are conventionally evaluated in rat models of drug dependence, such as conditioned place preference (CPP), sensitization in locomotor activity, and drug self-administration (Browning et al., 2011; Knutson et al., 1998; Maier et al., 2012; Meyer et al., 2012; Simola & Morelli, 2015; Taracha et al., 2016). In this regard, it is noteworthy that drugs that have abuse potential may elicit either aversive or rewarding effects in rats, and that these effects are not necessarily paralleled by the manifestation of behaviors that are thought to mark the presence of drug-induced modifications in arousal and emotional state (Verendeev & Riley, 2011). Accordingly, measuring the emission of 50-kHz USVs as a marker of positive affect may offer some notable advantages in rat studies of drug dependence, since this approach could reveal effects of drugs on the state of arousal and on the emotional state that may be not fully

disclosed by conventional behavioral readouts. Moreover, and like what has been reported for the emission of 22-kHz USVs, the emission of 50-kHz USVs may reveal how individual traits influence the affective properties of drugs, and eventually the development of addictive-like behaviors. This has been demonstrated in experimental models of amphetamine dependence, where the magnitude of sensitization in the emission of 50-kHz USVs elicited by passive and repeated administration of amphetamine predicted the subsequent acquisition of amphetamine self-administration (Taracha et al., 2016).

Of note, most studies of neuroscience and neuropharmacology that have evaluated the emission of USVs in rats have assumed that the occurrence of qualitative and/or quantitative changes in ultrasonic calling behaviour reflects the presence of alterations in emotionality and/or sociability in the emitting animals. Nevertheless, it cannot be ruled out that the exposure to pharmacological and/or stressful stimuli and the genetic background may also impact the physiological mechanisms that mediate the emission of USVs, and that such an effect could participate in the modifications in calling behaviour that have been demonstrated in rat models of brain disorders. As of today, the existence of pathological modifications in the laryngeal mechanisms that mediate the emission of USVs has been demonstrated in rat models of Parkinson's disease (PD) (Ciucci et al., 2007; Johnson et al., 2015), whereas little is known on how phonation mechanisms are affected in other models of brain disorders. Future studies aimed at addressing this issue will be of great relevance to fully understand the biological significance of the altered USV emissions that are observed in rat models of brain disorders.

3 | NEW PERSPECTIVES ON THE USE OF ULTRASONIC VOCALIZATIONS IN STUDIES OF NEUROSCIENCE AND NEUROPHARMACOLOGY IN MICE AND RATS

The evidence presented in the first part of this review demonstrates that USVs of mice and rats are of great interest as behavioral markers in preclinical studies of affectivity and sociability in brain health and disease. Indeed, measuring USVs may complement and refine the currently available experimental models, and potentially increase the amount of information that can be obtained in studies of neuroscience and neuropharmacology in mice and rats. The second part of this review will offer some new views and perspectives on the experimental use of measuring the emission of USVs in mice and rats, by providing an overview and discussion of the data presented during the Symposium '*Ultrasonic vocalizations in rodents: relevance for ethologic and pharmacologic studies*' held in the framework of the XIX National Congress of the Italian Society for Neuroscience.

3.1 | Advances in the automated classification of ultrasonic vocalizations

USVs are recorded using an ultrasound frequency sensitive microphone and later analysed with specific software. While the ultrasonic vocalization software is typically very useful for quantitative parameters analysis (number, duration, peak amplitude, peak frequency, etc.), their application for the qualitative analysis of calls is often more complicated. In fact, only few commercial software solutions have been developed to perform also the qualitative analysis of the USVs, yet by creating their own classification of the different types of calls, which may therefore not find a correct correspondence with the classifications most commonly used by the various research groups that study communication in rodents. Therefore, a manual analysis is often performed to classify the calls; this step of the analysis is highly time-consuming. Another disadvantage of manual classification of USVs is its dependency on the operator, so that the same person needs to analyse the entire dataset, to avoid biases linked to variations in the subjective interpretation of USVs' characteristics. However, the manual classification of USVs still allows for the most highly detailed characterization.

Hence, it is not surprising that several automatic classification systems have been created, including the MUPET system for mice (Van Segbroeck et al., 2017), the WAAVES system for rats (Reno et al., 2013), and DeepSqueak for various animal calls (Coffey et al., 2019). All these tools can automatically separate audio inputs into calls and background noise. They then use a classification algorithm to label the obtained vocalizations. WAAVES, for instance, employs a binary classification with two categories: frequency modulated calls and flat calls. MUPET and DeepSqueak, on the other hand, execute unsupervised clustering with a greater number of categories. MUPET can only cluster into a known a priori number of classes that must be determined by the user, whereas DeepSqueak can automatically discover the best-fitting number of clusters using statistical approaches. Furthermore, MUPET cannot apply its models to datasets other than those for which they were originally developed for, thus requiring separate models for each dataset. On the basis of a well-known pre-established set of call types, the USV classification pattern published by Scattoni,

Gandhy, et al. (2008), we have developed an ad hoc method for automatic USVs classification (Premoli, Baggi, et al., 2021). We began our classification by using an audio recording that had already been segmented into vocalizations and noise, with a dataset of 48,699 labelled segments. Our method is based on a top-down approach whereas the other methods are based on a bottom-up one (Ivanenko et al., 2020; Leonardi & Migliorati, 2002). We designed and employed two deep learning methods, a Convolutional Neural Network (CNN) and a Multilayer Perceptron (MP), as well as two machine learning techniques, that is, Support Vector Machines (SVM) and Random Forests (RF), in order to achieve an automatic classification task. The dataset was manually created using Avisoft software, which includes capabilities for visualizing and manually segmenting audio tracks in the form of spectrograms. The obtained USVs were then processed to extract some informative features required for training the learning algorithms. The results demonstrated that utilizing the entire time/frequency information of the spectrogram results in a better performance than taking into account only a subset of numerical features. The findings appear to be encouraging, and we anticipate they may offer a new standard for future research in this field.

3.2 | Ultrasonic vocalizations as a tool to assess the validity of mouse models of neurodevelopmental disorders: The example of maternal immune activation-based mouse models for ASD

In the context of NDDs, an interesting hypothesis has recently emerged: aberrant immune activity during critical periods of neurodevelopment could participate in the generation of neurological dysfunction characteristic of several NDDs. This immune system dysregulation during pregnancy is also called 'maternal immune activation' (MIA), and it has recently emerged as an important risk factor for several NDDs, including ASD and schizophrenia (Bilbo & Schwarz, 2009; Boksa, 2010; Harvey & Boksa, 2010; Meyer et al., 2011). Activation of the maternal immune system during pregnancy by infection through common viruses or bacteria has been linked to lifelong changes in brain function and behaviour of offspring (Jouda et al., 2019). A combination of genetic background, autoimmune status and second hits during childhood and adolescence (including stress and drug abuse) combines with the consequences of MIA to increase the likelihood of offspring developing NDDs or neuropsychiatric disorders as adults (Bilbo & Schwarz, 2009; Boksa, 2010; Estes & McAllister, 2016; Harvey & Boksa, 2012; Meyer et al., 2011). To better study the biological mechanisms at the basis of these effects, we used two different MIA murine models: the $p50^{-/-}$ mouse (genetic model) and the LPS-prenatal exposed mouse (induced model).

$p50^{-/-}$ mice present a deletion of the *Nfkb1* gene, coding for the p50 NF-κB pathway subunit. NF-κB is an important transcriptional factor that plays different roles in many cellular processes including regulation of neuronal survival and plasticity, inflammation, cell proliferation, neurogenesis, learning and memory (Boersma et al., 2011; Bonini et al., 2011; Grilli & Memo, 1999; Gutierrez & Davies, 2011; Mattson & Meffert, 2006; Pereira & Oakley, 2008; Zhang et al., 2012). Furthermore, $p50^{-/-}$ mice show multifocal defects in immune responses involving B lymphocytes and non-specific responses to infection (Sha et al., 1995). They present multifocal defects in immune responses and are characterized by chronic neuroinflammation (Bonini et al., unpublished data). In addition, $p50^{-/-}$ mice exhibit some abnormalities in structural plasticity and cortical structure alterations (Bonini et al., 2016). Concerning behaviour, $p50^{-/-}$ mice, in addition to an increase in locomotor and exploratory activity, display impairment in social behaviors, with a significant reduction in social interaction (Bonini et al., 2016). For all these reasons, we are studying $p50^{-/-}$ mouse as a new potential model of NDDs with an ASD-like phenotype. We have characterized the specific profile of ultrasonic communication in $p50^{-/-}$ mice on USV emission both in infancy and adulthood. In maternal separation-induced USVs, we found that WT pups emitted an inverted U-shaped call emission pattern that followed a typical ontogenetic profile of USVs mouse pups, and it was exacerbated in $p50^{-/-}$ pups. Furthermore, they emitted significantly more and longer USVs compared to wild-type pups. A detailed analysis displayed that WT pups emitted a quite homogenous repertoire of calls; instead, $p50^{-/-}$ pups emitted a different repertoire of calls, which included higher number of 'two syllable' and 'frequency steps' with a longer duration of these calls than WT pups (Premoli et al., 2019). During male–female social interaction test, adult $p50^{-/-}$ mice emitted decreased number of USVs than WT mice associated with a reduced social interaction, in particular reduced sniffing behaviors. We hypothesize that this reduced number of USVs found in adult $p50^{-/-}$ mice can be linked to reduced communication and social interaction typical of adult human patients with NDDs. Interestingly, the same categories of calls altered in $p50^{-/-}$ pups were reduced also in $p50^{-/-}$ adult mice. Therefore, alterations of ultrasonic communication found in $p50^{-/-}$ mice are not generalized but call-specific; indeed, only few types of calls are different in

comparison to those of WT mice. This could have an important role in the context of specific call meaning.

Lipopolysaccharide (LPS), a component of Gram-negative bacteria, is a typical stimulus used to mimic infection in many animal studies of MIA (Ortega et al., 2011; Urakubo et al., 2001), and the LPS-prenatal exposed mouse is a well characterized model of NDDs (Brown & Meyer, 2018; Patterson, 2011; Wischhof et al., 2015). Usually, LPS-induced MIA model are generated by administering the inflammatory stimulus acutely in a specific pregnancy window to study how inflammation affects a specific stage of embryonic development (Meyer et al., 2006, 2007). On the contrary, we decided to treat chronically dams with low doses (.3 mg/kg) of LPS during pregnancy and to study the LPS effects on offspring. This was done in order to simulate a situation of chronic inflammation during pregnancy and to study its long-term consequences. In particular, we analysed USV emission in pups during maternal separation. We found a significant increase in calls number at PND 8 compared to vehicle-treated pups emitting USVs (Aria et al., 2020). Furthermore, in order to better understand the biological mechanisms underlying the inflammatory response induced by LPS in the offspring, we treated dams with meloxicam, a well-known nonsteroidal anti-inflammatory drug cyclooxygenase-2 preferential throughout pregnancy. Then, we studied USVs in the offspring; it emerged that LPS-prenatally exposed pups of mother treated with meloxicam during pregnancy (LPS + MLX-pups) emitted significantly more USVs than LPS-pups. Additionally, looking at calls duration, meloxicam treatment induced a significant increase in duration of calls compared to vehicle- and to LPS-pups. No sex differences were found. Together, we demonstrated that a chronic LPS injection during pregnancy causes communication alteration in pups, and that a maternal meloxicam treatment, instead of preventing this alteration, exacerbates this abnormal vocalization pattern.

3.3 | Ultrasonic vocalizations as a tool to assess the validity of mouse models of neurodevelopmental disorders: The example of monogenic mouse models for ASD

As previously mentioned, the most «classical» settings used to evaluate USVs include isolation-induced USVs in mouse pups (during the first days of development, for example, PND 7–10) (Scattoni et al., 2009) and courtship-related USVs emitted by a male mouse during dyadic interactions with an adult female (Hammerschmidt et al., 2009; White et al., 1998). Recently, a novel interest has emerged towards female USVs that are mostly assessed during female–female interactions in a resident intruder setting, that is, following a 72hs pre-testing isolation of the resident (Maggio & Whitney, 1985; Moles et al., 2007). These «classical» approaches to the analysis of USVs have been applied in our recent studies on monogenic mouse models of ASD, in order to evaluate their face validity.

We have recently focused on two mouse models, the *CB1*-KO and the *Fmr1*-KO mouse lines, based on two opposite approaches (Oddi et al., 2013). The *CB1*-KO mouse line intends to model ASD through a 'bottom-up' approach, that is, targeting the major cannabinoid receptor CB1, hence manipulating a neurobiological system (the endocannabinoid system) that has been shown to be altered in ASD patients (Pietropaolo & Marsicano, 2022), in order to induce ASD-like symptoms. The *Fmr1*-KO mouse line models instead FXS that is the most common monogenic cause of ASD. Hence through a top-down approach, this model may allow to identify the neurobiological mechanisms involved in ASD etiopathology (Bernardet & Crusio, 2006; Pietropaolo et al., 2011).

For both genetic models, we have investigated USVs in pups and adults, including mice of both sexes. Despite a traditional focus on the male sex, ASD-research is indeed increasingly interested in evaluating pathological behavioral phenotypes also in female subjects, as ASD female patients may have unique clinical presentations relative to their male counterparts, a factor that may have led to under diagnosis of ASD in the female sex (Loomes et al., 2017). The relevance of including females in studies on ASD is also further supported by the importance of female carriers in X-related syndromes, such as FXS and other X-linked pathologies strongly associated with ASD.

For pups' evaluation, the analysis of developmental profiles, including the evaluation of USVs number, duration and peak-frequency and amplitude during multiple PNDs is another factor of crucial relevance. Several mouse models of ASD have shown no overall alterations in these parameters during the first postnatal week, but they were characterized by a day-dependent shift in their peak or decline. For adult USVs, a detailed qualitative analysis has become urgently needed; this includes the analysis of the call types through their classification based on spectrographic characteristics (Caruso et al., 2020; Premoli et al., 2019; Premoli, Memo, & Bonini, 2021) or their evaluation through density plots visualizing the spread of the calls according to their amplitude/frequency characteristics (Mosienko et al., 2015; Wöhr, 2014). Recent technological advances have made these qualitative analyses increasingly sophisticated (Ivanenko et al., 2020; Premoli, Baggi, et al., 2021), thus contributing to ameliorate our understanding

of the complexity of adult mouse ultrasonic communication and of its alterations in ASD models. Finally, playback studies have started being applied to evaluate USVs also from the point of view of the receiver and not only of the emitter (Hammerschmidt et al., 2009; Wöhr, Moles, et al., 2011), that is, a major aspect of assessing social communication in mammals. We have applied all these approaches to our two examples of ASD models and obtained findings that are highly complementary and allowed to draw some common conclusions on ultrasonic communication in ASD mouse lines.

3.4 | The ultrasonic profile of the CB1-KO mouse: A potential monogenic mouse model for ASD

The endocannabinoid system (ECS) is a highly promising candidate to study the etiopathology of ASD and to identify novel therapeutic targets. It is primarily a retrograde inhibitory signalling pathway that includes the primary cannabinoid type-1 receptor (CB1r) and the endogenous ligands, the endocannabinoids (Devane et al., 1992; Sugiura et al., 1995). ECS modulates neuronal functions, as demonstrated by the abundance of CB1 receptors in the brain (Mackie, 2005), and regulates synaptogenesis and neuronal interconnectivity (Berghuis et al., 2007; Mulder et al., 2008), that is, all processes that are known to be affected by ASD pathology (Pardo & Eberhart, 2007). Also, alterations in the expression of CB1r and other ECS components, as well as in their functionality, have been described in ASD patients (Karhson et al., 2018; Smith et al., 2017) and in several ASD animal models (reviewed by Zamberletti et al., 2017). Finally, recent clinical and preclinical studies supported the efficacy of pharmacological modulators of ECS as therapeutic approaches to ASD symptoms (Bar-Lev Schleider et al., 2019; Jung et al., 2012; Pretzsch et al., 2019).

Based on this large evidence, we therefore examined ultrasonic communication in male and female *CB1* null mutants, homo ($CB1^{-/-}$) or heterozygous ($CB1^{+/-}$) for the mutation, during development (i.e., between PNDs 4 and 10) and at adulthood (3 months). During the postnatal period both male and female $CB1^{-/-}$ pups showed altered day-dependent patterns of expression of several USV parameters. These included a developmental profile (Branchi et al., 2001; Sungur et al., 2016) characterizing the number and mean duration of calls produced by $CB1^{+/+}$ mouse pups, with a peak around PND 4–6, followed by a reduction on PNDs 8 and 10. A similar peak in USV rate and duration was displayed by $CB1^{-/-}$ pups, but it was not followed by a comparable reduction on the subsequent days. USV mean peak frequencies also followed a clear developmental pattern, with a linear increase from PND 4 to PND 10 that was markedly reduced in $CB1^{-/-}$ male and female pups. Furthermore, density plots revealed in $CB1^{+/+}$ pups the presence at PND 4 of a single cluster of lower mean peak frequency calls (50–70 kHz), associated at PND 6 and 8 with a second cluster of higher frequency calls (80–100 kHz) and disappearing on PND 10, when only the higher frequency cluster remained. In $CB1^{+/-}$ and $CB1^{-/-}$ pups both the high and low frequency clusters were instead still evident at PND 10, resembling the pattern observed in the *Shank1* mouse model of ASD (Sungur et al., 2016). These findings suggest a delay in the development of communication abilities in *CB1* mutants. Male and female $CB1^{-/-}$ mice showed again at adulthood both quantitative and qualitative USV alterations, including a reduction in the call rate, and higher variations in the peak frequency and mean duration of the calls (i.e., reduced clustering), as revealed by the density plots.

Our findings on neonatal USVs point out to a developmental delay in $CB1^{-/-}$ pups, since the day-dependent reduction of USV rate is supposed to correspond to increased pup maturity and reduced dependence from the mother (Caruso et al., 2020). Furthermore, previous studies have shown that USV rate in rodent pups reflect their affective state (Branchi et al., 2001; Zimmerberg et al., 1994), with an increase corresponding to an adverse emotional state (Caruso et al., 2020; Oddi et al., 2015). The increased pups' USV rate, accompanied by qualitative alterations in spectrographic parameters, such as mean duration or peak frequency, it has also been interpreted as a sign of altered communication abilities, within the context of mother-infant interactions. Interestingly, infants with ASD or at risk, when compared to typically developing infants, emit more cries with certain specific spectrographic characteristics (e.g., higher fundamental frequencies), which have been linked to more aversive conditions and negatively affect parental responses (Esposito et al., 2017; Esposito & Venuti, 2010).

These findings add to previous evidence showing that CB1 inhibition in mice induces alterations relevant to ASD core symptoms, such as repetitive and inflexible behaviors (Gomes et al., 2011; Pietropaolo et al., 2020; Terzian et al., 2011; Varvel & Lichtman, 2002) and social deficits (reviewed in Pietropaolo & Marsicano, 2022). Hence, our results further support the potential role of the ECS in the etiopathology of ASD and therefore its relevance as a therapeutic target for ASD-related pathologies. Our data also support the validity of the *CB1* null mouse line as a potential novel monogenic mouse model for ASD, useful not only for preclinical studies on ASD but also for studies on the neurobiological mechanisms underlying the general control of social behaviors and communication.

3.5 | The ultrasonic profile of the Fmr1-KO mouse: A validated monogenic mouse model for ASD

Fragile X syndrome (FXS) is a pervasive developmental disorder caused by expansion of a CGG repeat in the promoter region of the X-linked FMR1 gene (Pieretti et al., 1991), causing gene silencing and subsequent deficiency of the encoded Fragile X Mental Retardation Protein (FMRP) (Oberle et al., 1991). As an RNA-binding protein, FMRP plays a key role in controlling synaptic functionality and maturation (Santoro et al., 2012); its lack induces a variety of behavioral alterations in FXS patients (Tsiouris & Brown, 2004), including hyperactivity, increased anxiety, sensory hyper-responsiveness, cognitive deficits and abnormalities in social behaviors. These symptoms overlap with many of the behavioral deficits observed in ASD (Bailey et al., 1998; Bailey et al., 2001; Brock & Hatton, 2010; Rogers et al., 2001) and the two pathologies are intertwined at the molecular level (Darnell et al., 2011; Parikshak et al., 2013). Since all FXS males meet some aspects of the DSM criteria for ASD and approximately 50%–80% meet the full criteria (Roberts et al., 2007), increased research interest has been devoted to the common mechanisms underlying FXS and ASD pathologies, thus prompting an interest in using FXS mouse models, that is, the *Fmr1*-KO lines (Mientjes et al., 2006; The Dutch–Belgian Fragile X Consortium, 1994), to study ASD (Bernardet & Crusio, 2006; Oddi et al., 2013; Pietropaolo & Subashi, 2014).

In contrast to the *CB1*-KO mouse line, the *Fmr1*-KO model has been extensively characterized for its ASD-like phenotypes (for reviews, see Oddi et al., 2013; Pietropaolo & Subashi, 2014) and their possible correction by pharmacological (Busquets-Garcia et al., 2014; Silverman & Crawley, 2014) and environmental interventions (Oddi et al., 2015; Restivo et al., 2005). In the last decade, its ultrasonic profile has been also investigated, both during early life phases and at adulthood, leading to some discrepant results, mainly due to the use of different backgrounds to implement the Fmr1 mutation (Belagodu et al., 2016; Hodges et al., 2017; Nolan et al., 2020; Reynolds et al., 2016; Rotschafer et al., 2012; Roy et al., 2012; Spencer et al., 2011; Toledo et al., 2019). We focused all our studies on the C57Bl/6 common background and we evaluated pup, adolescent and adult USVs in *Fmr1*-KO males ($^{-/Y}$) and heterozygous females ($^{+/-}$) and compared their qualitative and quantitative ultrasonic characteristics with those of their WT littermates (Gaudissard et al., 2017; Gauducheau et al., 2017; Oddi et al., 2015; Petroni et al., 2022).

We demonstrated that *Fmr1*-KO male mice showed qualitative alterations in ultrasonic communication during the first postnatal week: KO pups emitted longer USV calls compared to WT when the day-by-day profile was examined (but not in a study when only PND 8 was considered; Oddi et al., 2015). These alterations disappeared at adolescence (Gaudissard et al., 2017; Petroni et al., 2022) and adulthood (Gaudissard et al., 2017; Oddi et al., 2015); nonetheless, while adult WT male mice emitted longer USVs compared to WT adolescents, this qualitative change was absent in adult KO males (Gaudissard et al., 2017), thus suggesting a deficit in the maturation of this social behaviour.

When we examined *Fmr1*-KO heterozygous females we found USVs alterations depending on the testing age. No differences (either quantitative or qualitative) were observed at infancy (Gauducheau et al., 2017), while at adolescence mutant females showed an increase in the number of USVs as well as in their duration (Gauducheau et al., 2017; Petroni et al., 2022). These abnormalities disappeared at adulthood, except for the increase in USV duration that persisted at the later age (Gauducheau et al., 2017). While the analysis of call subtypes revealed no major difference in the composition of the calls emitted by mutant males, female *Fmr1*-KO emitted less simple calls, that is, based on one or two components [and more complex calls, that is, containing 3, 4, 5 or more components, than their WT littermates] (Petroni et al., 2022). This result obtained from call classification is in line with the findings obtained from the density plots (Petroni et al., 2022) showing a prevalence of long calls in female mutants, since complex calls typically correspond to longer USVs.

Our findings from *Fmr1* male and female mutants suggest that infancy is the less sensitive phase to the effects of the *Fmr1* mutation, with light qualitative differences only being observed. It is interesting to underline that quantitative differences were rarely observed, and only in female *Fmr1* mutants. When these differences were detected, they consisted of an increased USV rate. This finding is not unusual, since it has been reported in this (Spencer et al., 2011) and other mouse models of ASD (Jiang et al., 2010; Nakatani et al., 2009; Scattoni, Gandhy, et al., 2008; Schmeisser et al., 2012; Tsai et al., 2012). Although a first interpretation of clinical data would have translated the communication deficits of autistic FXS patients into a reduction in the number of USVs emitted by *Fmr1* mutant mice, it should be noted that several studies describe excessive talking and repetitive speech as major ASD-like signs of altered communication in FXS patients (see, for e.g., Wheeler et al., 2014). In this light, the more abundant and longer USVs emitted by *Fmr1* mutant males and females at specific ages may mimic the human situation.

The absence of marked abnormalities in infancy in *Fmr1* mutants is in contrast with what is observed in

humans (see, e.g., Clifford et al., 2007). This discrepancy may be due to species-specific differences in the effects of the genetic mutation but could also be a side-effect of the restricted behavioral repertoire of mouse pups, due to the remarkably higher behavioral immaturity of newborn mice compared to humans. Indeed, USV assessment is almost the only behavioral tool available to investigate FXS- and ASD-like deficits in mouse pups (Ricceri et al., 2007), since cognitive and social abilities are not developed yet. The minimal alterations detected at infancy in the *Fmr1*-KO mouse model also suggest that early postnatal ages are the most encouraging targets for interventions aimed to prevent the appearance of social ASD- and FXS-like deficits, especially in female carriers, an issue that is obviously highly relevant for the development of therapeutic strategies.

In conclusion, our results underline the relevance of age as a critical factor influencing the face validity of the *Fmr1*-KO model for ASD and should therefore be taken carefully into account in future studies. Apart from the limitations of infancy, our ultrasonic studies support the relevance of the adolescent age, as a phase of interest to investigate ASD-like and communicative alterations. This age is indeed a well-known sensitive period for brain and behavioral plasticity in the mouse species as well as in humans (Spear, 2000).

3.6 | Common aspects of the two mouse models: Conclusions and perspectives

The comparison of the USV findings obtained from these two monogenic mouse models for ASD, the *CB1*-KO and the *Fmr1*-KO mouse lines, highlight some common important points, despite the specific differences in the age- and sex-dependent ultrasonic profiles.

The first common point lies in the limited absolute relevance of quantitative differences to model ASD-like features: no changes (in *Fmr1*-KO pups), decrease (as in *CB1*-KO at adulthood), or increase (in *CB1*-KO pups and *Fmr1*-KO juveniles) in USV rates have been described, in line with previous literature reporting reduced (Ey et al., 2011; Rotschafer et al., 2012; Scattoni, Gandhy, et al., 2008; Scattoni, McFarlane, et al., 2008; Schmeisser et al., 2012; Silverman et al., 2010) or enhanced (Jiang et al., 2010; Nakatani et al., 2009; Scattoni, Gandhy, et al., 2008; Schmeisser et al., 2012; Tsai et al., 2012) USV emission as a translationally relevant ASD-like phenotype in several ASD mouse models. Our data support the view that quantitative alterations in USV emission should be carefully interpreted in the specific testing context, also comparing the effects of positive or aversive manipulations on this behavioral endpoint. We have for instance demonstrated that exposure to an enriched environment reduces the number and duration of USVs in both WT and Fmr1 mutants, thus suggesting a 'negative' value of increased rates and duration in this specific mouse line.

Another common point raised by both the *CB1*-KO and the *Fmr1*-KO models is the relevance of qualitative alterations to model ASD-like communicative features. In particular, a longer duration of the USVs, sometimes accompanied by a higher complexity of the calls (at least in female mutants), seems the most important characteristic of USVs with relevance to ASD. Since several studies have described excessive talking and repetitive speech as major autistic alterations (see, e.g., Wheeler et al., 2014), it is possible that longer USVs may have less appropriate or adaptive communicative properties. Our preliminary unpublished findings from playback studies seem to support this conclusion (Pietropaolo: personal communication, SINS 2021), but more data are needed to extensively evaluate the communicative meaning of longer and more complex calls and their impact on male and female mouse behaviour.

Finally, our findings from the two mouse models underline the importance of including female mice in ASD studies. Since several and even more varied ASD-like phenotypes were detected in female *CB1* and *Fmr1* mutants, it is evident that females are equally valuable for the investigation of the etiopathological mechanisms of ASD and FXS and to identify potential treatments. Our results also demonstrate that the possible behavioral variations induced by the estrous cycle do not limit the validity of female mouse studies; although we found differences in the estrous phase among experimental subjects, these were similarly distributed between WT and *Fmr1* or *CB1* mutant females and they did not affect their ultrasonic profiles (Fyke et al., 2021; Gauducheau et al., 2017; Petroni et al., 2022). Hence, differences in ultrasonic characteristics due to the considered mutant genotypes could not be biased by estrous differences nor were our findings specific of a single estrous phase.

4 | RAT 50-kHz ULTRASONIC VOCALIZATIONS AS A NOVEL BEHAVIORAL MARKER IN PRECLINICAL STUDIES OF THE AFFECTIVE PROPERTIES OF DOPAMINOMIMETIC DRUGS USED IN THE TREATMENT OF PARKINSON'S DISEASE

An altered emission of USVs has been described in diverse rat models of neurological diseases. Reductions in the numbers of 40-kHz USVs emitted in response to

maternal isolation and in time spent calling have been demonstrated in rat pups subjected to cerebral ischemia (Saucier et al., 2008). Besides, increased emission of 22-kHz USVs in response to the delivery of air puffs has been reported in rats subjected to traumatic brain injury (Browning et al., 2017). These findings indicate that the emission of USVs may be a relevant behavioral marker to be evaluated in rat models of cerebral ischemia and brain trauma. Nevertheless, it is still to be determined whether altered ultrasonic calling behaviour in these experimental models reflects modifications in emotionality, vocal functions, or in both domains. Moreover, a very recent study has shown that the emission of either 22-kHz USVs or 50-kHz USVs may accompany the manifestation of seizures elicited by the administration of pentylenetetrazol in rats, suggesting that USVs could be a novel behavioral marker to be evaluated in rat models of seizures and epileptiform activity (Lara-Valderrábano et al., 2022). Furthermore, the emission of 50-kHz USVs has been extensively investigated in rat paradigms of PD. Although USVs of rats cannot be considered equivalent to human language (Brudzynski, 2005), as detailed earlier in this review, rats generate ultrasonic calls through the larynx, a process similar to the production of human voice (Riede et al., 2017). Accordingly, it has been proposed that evaluating the qualitative and quantitative changes of the 50-kHz USVs that are emitted by dopamine-denervated rats may be a relevant approach to model at the preclinical level the alterations in vocal function that may affect parkinsonian patients (Johnson et al., 2015). This hypothesis is corroborated by the evidence that rats bearing a PD-like hypofunction of the dopaminergic nigrostriatal system display a reduced complexity of the emitted 50-kHz USVs, consisting in an increased emission of flat calls at the expenses of FM calls, and in the acoustic degradation of the emitted calls, which display a decrease in maximum sound frequency and bandwidth (Ciucci et al., 2007; Ciucci et al., 2009; Grant et al., 2015). Indeed, the acoustic degradation of 50-kHz USVs that occurs in rat models of PD mimics, to some extent, the reductions in sound bandwidth and amplitude featuring the vocal dysfunction that may be manifested by PD patients (Johnson et al., 2015).

The available studies that have demonstrated the presence of alterations in ultrasonic calling behaviour in rat models of neurological diseases have evaluated the emission of USVs in response to non-pharmacological stimuli. Relatively limited information, however, is available on the effects that pharmacological stimuli may have on ultrasonic calling behaviour in rat models of neurological diseases. Most notably, Simola and colleagues have recently explored the possibility that the emission of 50-kHz USVs may be a relevant behavioral marker to be evaluated in preclinical investigations about the affective properties of dopaminomimetic drugs that are used in the management of PD (Simola et al., 2021).

The pharmacotherapy of PD consists of the so-called 'dopamine replacement therapy' (DRT), which relies on the dopamine precursor L-3,4-dihydroxyphenilalanine (L-DOPA) and other drugs that potentiate dopaminergic transmission in the nigrostriatal system (Pinna et al., 2018). The use of DRT effectively counteracts the motor impairment featuring PD but may be associated with complications involving the motor and non-motor domains (Morelli et al., 2012; Voon et al., 2017). Among the latter are iatrogenic psychiatric-like disturbances, that include impulsive-compulsive behaviors, psychotic-like symptoms, and the so-called 'dopamine dysregulation syndrome', which consists in the addictive-like use of dopaminergic medications (Voon et al., 2017). The management of iatrogenic psychiatric-like disturbances in PD patients is hampered by the lack of effective therapeutic strategies. This may depend, at least in part, on the paucity of preclinical models that exhaustively reproduce the iatrogenic psychiatric-like disturbances displayed by PD patients and allow the study of the underlying neurobiological mechanisms. Drugs used in the DRT of PD may modify the emotional state in humans and experimental animals (Campbell et al., 2014; Engeln et al., 2013; Kapogiannis et al., 2011; Riddle et al., 2012). Moreover, PD patients with iatrogenic psychiatric-like disturbances may display alterations in their affective state (Delpont et al., 2017; Terenzi et al., 2018). In consideration of this evidence and of the interplay existing in rats between modifications in the affective state and changes in ultrasonic calling behaviour, it was hypothesized that rat USVs could be a relevant behavioral marker to be evaluated in preclinical investigations aimed at elucidating the affective properties of drugs used in the DRT of PD. To address this issue, a study was performed in rats rendered hemiparkinsonian by means of a unilateral lesion of the nigrostriatal system with the neurotoxin 6-hydroxydopamine (6-OHDA), the most widely used rat model of PD (Simola et al., 2007), that were thereafter repeatedly treated with apomorphine, L-DOPA or pramipexole, three drugs that are used in the DRT of PD and may elicit psychiatric-like disturbances in patients (Todorova et al., 2015; Vargas et al., 2019; Weintraub et al., 2015). Pharmacological treatments were performed according to a previously validated protocol (Costa et al., 2015; Simola et al., 2016; Simola & Morelli, 2015) that allows to evaluate different aspects of ultrasonic calling behaviour that are thought to reflect the presence of changes in the affective state of rats treated with psychoactive drugs, such as (i) emission of USVs immediately after drug administration, indicative of the acute

rewarding effects of drugs (Burgdorf et al., 2001; Simola et al., 2012); (ii) sensitization in calling behaviour, indicative of increased positive affect elicited by repeated drug exposure (Ahrens et al., 2009; Simola et al., 2014); (iii) calling behaviour elicited by the re-exposure to environmental cues previously paired with drug administration, indicative of environmental conditioning to the affective properties of drugs (Costa et al., 2020; Ma et al., 2010; Simola & Morelli, 2015).

Simola and colleagues reported two major findings: (i) different drugs used in the DRT of PD may elicit distinct effects on the emission of 50-kHz USVs in rats; (ii) the stimulation of ultrasonic calling behaviour by drugs used in the DRT of PD was more evident in hemiparkinsonian rats than in their sham-operated counterparts. Of the three dopaminomimetic drugs evaluated, only apomorphine significantly stimulated the emission of 50-kHz USVs in both hemiparkinsonian and sham-operated rats, although this effect was more pronounced in the former animals, which also displayed sensitization in calling behaviour with repeated drug administration. Conversely, L-DOPA stimulated the emission of 50-kHz USVs in hemiparkinsonian rats only and pramipexole had a negligible effect on calling behaviour, although hemiparkinsonian rats treated with pramipexole emitted more 50-kHz USVs than sham-operated rats treated with pramipexole. Moreover, hemiparkinsonian rats previously treated with either apomorphine or L-DOPA emitted 50-kHz USVs in response to the presentation of environmental cues that were earlier paired with drug administration. The latter finding suggests that apomorphine and L-DOPA may elicit environmental conditioning to their subjective properties in a rat model of PD (see Ma et al., 2010; Simola & Morelli, 2015). Several lines of evidence indicate that the emission of 50-kHz USVs in response to the administration of psychoactive drugs marks the presence of drug-induced reward (Burgdorf et al., 2001; Simola & Brudzynski, 2018a). Accordingly, the results obtained by Simola and colleagues indicate that measuring the emission of 50-kHz USVs may be a relevant approach to investigate how the degeneration of the dopaminergic nigrostriatal system modifies the affective properties of drugs that are used in the DRT of PD. Indeed, the heightened emission of 50-kHz USVs that was found in hemiparkinsonian rats treated with either apomorphine or L-DOPA could indicate that dopaminergic nigrostriatal denervation enhances the sensitivity of rats to the positive effects that drugs used in the DRT of PD elicit on the emotional state. The results obtained by Simola and colleagues may also suggest that the denervation of the dopaminergic nigrostriatal system has a long-term boosting influence on the affective properties of drugs that are used in the DRT of PD. Thus, hemiparkinsonian rats, but not sham-operated rats, that were treated with either apomorphine or L-DOPA not only emitted 50-kHz USVs in response to environmental cues previously paired with pharmacological treatment (see above), but also displayed sensitization in the emission of 50-kHz USVs during repeated pharmacological treatment. Indeed, earlier studies performed in rats treated with drugs of abuse have suggested that both these effects may indicate the presence of a persistent elevation in the affective state (Ahrens et al., 2009; Simola et al., 2014). Furthermore, the results obtained by Simola and colleagues propose that measuring the emission of 50-kHz USVs may be a useful approach to study at the preclinical level how the occurrence of modifications in the affective state modulates the behavioral effects that are elicited by the dopaminomimetic drugs used in the DRT of PD. In this regard, it is noteworthy that apomorphine, L-DOPA and pramipexole elicited effects on calling behaviour in hemiparkinsonian rats that displayed either similarities or differences when compared with the effects that the same drugs elicited in dopamine-denervated rats that were tested in the CPP paradigm, which evaluates the presence of drug-mediated conditioned reward (Bardo & Bevins, 2000). Thus, the ability of apomorphine to stimulate the emission of 50-kHz USVs in hemiparkinsonian rats is consistent with earlier findings showing that apomorphine elicits CPP in the same rat model of PD (Campbell et al., 2014). Conversely, the effects of L-DOPA and pramipexole on the emission of 50-kHz USVs in hemiparkinsonian rats diverge from the results previously obtained in studies of CPP, since experiments in rats bearing a bilateral dopaminergic denervation of the nigrostriatal system found that pramipexole induced CPP (Riddle et al., 2012), whereas L-DOPA did not (Carvalho et al., 2017; Zengin-Toktas et al., 2013). Taken together, these findings may suggest that measuring the emission of 50-kHz USVs could reveal the existence of modifications in the emotional state of rats treated with drugs used in the DRT of PD that may be not fully disclosed by other behavioral readouts that are conventionally evaluated in rat models of PD.

Nevertheless, some additional considerations are necessary to elucidate the interplay among ultrasonic calling behaviour, alterations in the emotional state and behavioral effects that drugs used in the DRT of PD elicit in rat models of PD. First, differences in the experimental procedures used (i.e., performance of unilateral or bilateral dopaminergic denervations, see Roedter et al., 2001) could underlie the dissimilarities in 50-kHz USV emissions and behaviour in the CPP paradigm that independent studies have observed in rats that carried a PD-like denervation of the nigrostriatal dopaminergic system and were treated with drugs used in the DRT of

PD. Moreover, previous studies of CPP performed in rats treated with drugs of abuse have shown that a dissociation may exist between the preference for the drug-paired compartment and the emission of 50-kHz USVs (Knutson et al., 1999; Taracha et al., 2014; Wright et al., 2012). Furthermore, other investigations have demonstrated that the receptor binding profile critically regulates the effects that dopaminomimetic drugs elicit on ultrasonic calling behaviour in rats. Indeed, a marked elevation in the emission of 50-kHz USVs has been observed after the administration of drugs that non-selectively stimulate D_1 and D_2-like receptors (Simola et al., 2012; Williams & Undieh, 2010), but not after the administration of drugs that are selective agonists of either dopamine receptor subtype (Scardochio & Clarke, 2013; Williams & Undieh, 2010). The latter considerations may explain why pramipexole, which is a selective agonist of D_2-like receptors, elicited negligible effects on calling behaviour in hemiparkinsonian rats, whereas apomorphine and L-DOPA, that produce the stimulation of both D_1 and D_2-like receptors, significantly increased the emission of 50-kHz USVs in hemiparkinsonian rats.

The study by Simola and colleagues adds further evidence to the usefulness of measuring the emission of USVs as a behavioral marker in studies of neuropharmacology that employ rat models, by suggesting that measuring the emission of 50-kHz USVs may be useful for characterizing at the preclinical level the effects that drugs used in the DRT of PD elicit on the emotional state. Addressing this issue may be a key step in understanding how the affective properties of drugs used in the DRT of PD may favour the manifestation of behaviors that reproduce in rat models the iatrogenic psychiatric-like disturbances that may occur in parkinsonian patients.

5 | USE OF ULTRASOUND PLAYBACK IN THE STUDY OF SOCIO-AFFECTIVE COMMUNICATION DEFICITS IN RAT MODELS FOR NEURODEVELOPMENTAL DISORDERS

There is robust evidence in support of the idea that 22-kHz and 50-kHz USVs serve important socio-affective communicative functions (Brudzynski, 2021; Wöhr, 2018). For example, playback studies revealed that 22-kHz and 50-kHz USVs evoke distinct sets of behavioral responses in recipient rats. While 22-kHz USVs, which are typically emitted in aversive situations, lead to behavioral inhibition in recipient rats (Brudzynski & Chiu, 1995; Fendt et al., 2018), 50-kHz USVs, which are typically emitted in appetitive situations, evoke increased levels of social exploratory behaviour, primarily oriented towards the sound source, that is, social approach behaviour, both in males (Wöhr & Schwarting, 2007) and females (Willadsen et al., 2014), sometimes paralleled by the emission of response calls (Berz et al., 2022; Olszyński et al., 2021). This suggests that 22-kHz USVs serve as alarm calls, whereas 50-kHz USVs function as social contact calls, consistent with the emotional valence of the situation where 22- and 50-kHz USVs are primarily emitted.

Behavioral responses elicited in recipient rats are associated with changes in heart rate and brain activation patterns. For example, it was found that aversive 22-kHz USVs induce an abrupt decrease in heart rate, while heart rate is increased in response to appetitive 50-kHz USVs (Olszyński et al., 2020). At the level of the brain, distinct activation patterns are evident. For example, in line with an alarming function, c-fos studies revealed that 22-kHz USVs activate the amygdala (Ouda et al., 2016; Sadananda et al., 2008), where 22-kHz USVs elicit tonic increases in firing rates, as demonstrated by electrophysiological studies (Parsana et al., 2012). In contrast, in line with an affiliative function, 50-kHz USVs cause tonic decreases in firing rates in the amygdala (Parsana et al., 2012), but lead to activation in the nucleus accumbens (Pultorak et al., 2016; Sadananda et al., 2008), where they elicit phasic dopamine release (Willuhn et al., 2014). Phasic dopamine release in the nucleus accumbens is strongly linked to reward processing (Ikemoto & Panksepp, 1999). Consistent with a rewarding nature of 50-kHz USVs, it was shown that rats self-administer 50-kHz USVs (Burgdorf et al., 2008), a behaviour dependent on the subthalamic nucleus (Vielle et al., 2021).

Together, this shows that 50-kHz USVs induce strong behavioral responses and associated changes in brain activation patterns in line with important socio-affective communicative functions as social contact calls. This suggests that social approach behaviour induced by playback of 50-kHz USVs may serve as a sensitive and valid measure for revealing socio-affective communication deficits in rodent models for neuropsychiatric dysfunctions, most notably NDDs, such as ASD. In a recent series of studies, this idea was tested in rats exposed to genetic or environmental risk factors. Detailed quantifications of behavioral responses elicited by playback of 50-kHz USVs were performed and the response pattern displayed by the rat model was compared to littermate controls.

Through this means, it was shown that genetic rat models for ASD and related NDDs display reduced social approach behaviour in response to 50-kHz USV playback. This includes *Shank3*-deficient rats (Berg et al., 2018). In

humans, *SHANK3* mutations lead to Phelan-McDermid syndrome and are one of the most penetrant causes of ASD (Betancur & Buxbaum, 2013). When exposed to playback of 50-kHz USVs, rats of all experimental conditions displayed an increase exploratory behaviour irrespective of genotype. During 50-kHz USV playback, exploratory behaviour was clearly directed towards the sound source of 50-kHz USVs and social approach behaviour was evident in all genotypes. However, in the minutes following playback, heterozygous *Shank3* mutant rats and wildtype littermate controls kept showing a preference for the sound source but not male homozygous *Shank3* mutant rats. This indicates that male homozygous *Shank3* mutant rats did not keep searching for a conspecific, while female homozygous *Shank3* mutant rats and rats of all other experimental conditions did, suggesting mild and sex-dependent socio-affective communication deficits caused by *Shank3* deficiency. Emission of response calls was not affected (Berg et al., 2018).

More prominent socio-affective communication deficits were evident in *Ube3a*-deficient rats (Berg, Petkova, et al., 2021). Maternal *UBE3A* deletions cause Angelman syndrome, a rare neurodevelopmental disorder characterized by intellectual disability, most notably lack of language acquisition, but with a happy, excitable demeanour and easily provoked laughter (Bird, 2014). Consistent with frequent laughter in humans, *Ube3a* maternal deletion rats displayed excessive emission of laughter-like 50-kHz USVs when rough-and-tumble play is mimicked through tickling (Berg, Jami, et al., 2021). During 50-kHz USV playback, however, the behavioral responses evoked were comparatively weak. Both, social exploratory behaviour as well as the social approach response were clearly lower in *Ube3a* maternal deletion rats, as compared to wildtype littermate controls (Berg, Pride, et al., 2020). Emission of response calls was not affected (Berg, Jami, et al., 2021). An attempt to rescue the socio-affective communication deficits displayed by *Ube3a* maternal deletion rats through systemic administration of the insulin-like growth factor-2 (IGF-2) did not improve the phenotype but caused a lack of social approach behaviour in wildtype littermate controls (Berg, Petkova, et al., 2021).

Likewise, *Cacna1c* haploinsufficient rats display deficits in social approach behaviour evoked by 50-kHz USV playback. *CACNA1C* is a cross-disorder risk gene strongly implicated in multiple neuropsychiatric dysfunctions, including ASD and bipolar disorder (Kabir et al., 2016). *Cacna1c* haploinsufficient rats were found to emit fewer 50-kHz USVs in a broad variety of conditions with high emotional valence (for a detailed overview on other relevant phenotypes caused by *Cacna1c* haploinsufficiency in rats, please see Wöhr et al., 2021). This includes social conditions, such as rough-and-tumble play during juvenility (Kisko et al., 2018) and social interaction behaviour in adulthood (Redecker et al., 2019), but also non-social conditions, most notably an appetitive Pavlovian conditioned approach task (Sangarapillai et al., 2022). Effects of *Cacna1c* haploinsufficiency on 50-kHz USV emission appears to be modulated by sex, for example, reduced 50-kHz USV emission during rough-and-tumble play is

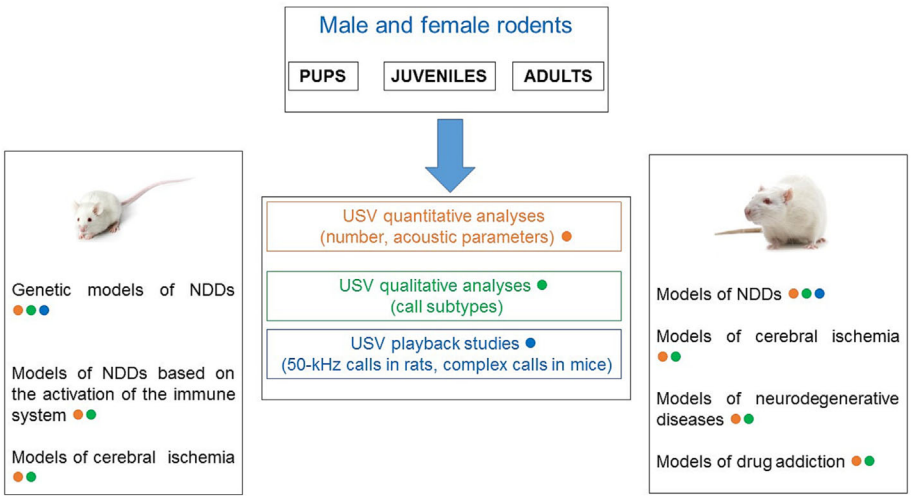

FIGURE 1 Overview on exemplary applications of measuring the emission of ultrasonic vocalizations and conducting playback experiments in mouse and rat models of brain disease. The analyses/procedures most commonly performed in studies that evaluate the emission of ultrasonic vocalizations in mice and rats are distinguished in different categories by three different colours (orange, green and blue). The application of these different types of analyses to different classes of experimental models of brain disease is visualized through the respective colours in mice (on the left) and rats (on the right). NDDs = neurodevelopmental disorders; USV = ultrasonic vocalization.

primarily seen in males (Kisko et al., 2020; for detailed acoustic analyses, please see: Kisko et al., 2021). This indicates that *Cacna1c* haploinsufficiency has detrimental effects on socio-affective communication in the sender. However, *Cacna1c* haploinsufficiency also affected the receiver. When focusing on recipient rats, *Cacna1c* haploinsufficient rats displayed less social approach behaviour in response to playback of 50-kHz USVs than wildtype littermate controls, albeit social exploratory behaviour was unchanged. This effect was primarily seen in male rats (Kisko et al., 2018; Kisko et al., 2020). Emission of response calls was also affected by *Cacna1c* haploinsufficiency in a sex-dependent manner (Kisko et al., 2020). Of note, behavioral inhibition evoked by playback of 22-kHz USVs was mildly reduced in male but not female *Cacna1c* haploinsufficient rats, suggesting that the effects of *Cacna1c* haploinsufficiency are modulated by sex across a broad range of social conditions, including sender and receiver (Wöhr et al., 2020).

In addition to genetic risk factors, there is first evidence suggesting that environmental risk factors reduce social approach behaviour in response to playback of 50-kHz USVs as well. This includes early life exposure to the organophosphorus pesticide chlorpyrifos (Berg, Ching, et al., 2020) and social isolation during juvenility (Seffer et al., 2015).

There is a continued need for improving animal research on neurobiological mechanisms underlying neuropsychiatric dysfunctions (Homberg et al., 2021). Available evidence from playback studies in rats exposed to genetic or environmental risk factors suggests that social approach behaviour induced by playback of 50-kHz USV may serve as a novel marker for socio-affective communication deficits in relevant rodent models, in line with the recent recommendations for behavioral studies in rodent models for ASD (Silverman et al., 2022).

6 | CONCLUSIONS

The emission of USVs is a central aspect of the behavioral repertoire of mice and rats, with high ethological value. Taking emission of USV into account and applying a more ethological-valid experimental approach is likely to have beneficial effects on translational research. It may contribute to refine current experimental rodent models to study emotionality and sociability, and may advance our understanding on how brain disorders alter these domains and sensory systems.

The overview provided here strongly supports the idea that USVs of mice and rats can serve as valuable tool with high relevance for preclinical research in neuroscience and neuropharmacology (as schematized in Figure 1). The applications of USV analyses span across the neuroscience and neuropharmacology fields and include the study of brain disorders with their complex symptomatology. Furthermore, the study of USVs in mice and rats has proven to be a valuable tool for investigating those brain disorders that are characterized by communication deficits. Finally, measuring USVs represents a non-invasive tool to acquire data about the health status of the animals and the effects of treatments (pharmacological and non-pharmacological) in mouse and rat models of brain disorders. The importance of measuring USVs of mice and rats in studies of neuroscience and neuropharmacology can be summarized as follows:

1. The evidence reviewed here underlines that despite the main applications of USVs analyses are used in the field of NDDs, in particular ASD, USV analyses can be also useful to investigate other brain disorders, such as neurodegenerative diseases (e.g., PD) and cerebral ischemia.
2. The findings summarized here also highlight the importance of studying USVs in female rodents. It is increasingly accepted that brain disorders have to be studied in animals of both sexes, and that the occurrence of certain symptoms in female animals may have been under-diagnosed. Furthermore, the analysis of USVs in females may be highly informative because females display in some situations a richer USV repertoire and higher call complexity than males. A longitudinal analysis of USVs including early postnatal, adolescent/juvenile and adult phases emerges also as an important element supported by the most recent evidence obtained from the several mouse and rat models reviewed here. Subtle alterations can indeed be identified in rodent models of brain disorders by evaluating changes in several USV characteristics across the first PNDs or during adolescence. Obviously, in order to allow performing this complex assessment of mouse and rat USVs, including both sexes and multiple ages, the further development of automatic systems for USV analysis is needed, to reduce the highly-time consuming and operator-dependent limitations of current manual methods.
3. Finally, the findings reviewed here highlight the crucial relevance of studying USVs in mice and rats not only from the side of the emitter, but also of the receiver. The USV playback method allows to study the consequences of an ultrasonic acoustic signal on rodent behaviour. This will allow to better understand the role of certain types of calls and the 'meaning' of the message transmitted (at least in terms of its

positive or negative emotional valence). These playback approaches have been primarily performed in rats, typically using the 50-kHz calls, but have recently started to be applied also in mouse models, for instance by comparing the behavioral impact of complex versus simple calls. These studies need to be further developed to clarify the biological role and significance of USVs also in mice, as already done in rats. As the mouse is the most widely used animal for in vivo preclinical studies, a more in-depth study of the structure of USVs of mice in terms of 'syntax' and behavioral impact is a major objective of future basic research on brain function and disease.

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MP, SP, MW, NS and SAB: Writing–original draft and writing–review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

For the preparation of this review, scientific articles published in peer-reviewed journals on the PubMed database were taken in consideration using the following keywords: animal models, neurological disorders, neuropharmacology, neuropsychiatric disorders, ultrasonic playback and various combinations of the above terms.

PEER REVIEW

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