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Rat ultrasonic vocalizations and behavioral neuropharmacology: from the screening of drugs to the study of disease

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Abstract

Several lines of evidence indicate that rats emit ultrasonic vocalizations (USVs) in response to a wide range of stimuli that are capable of producing either euphoric (positive) or dysphoric (negative) emotional states. On these bases, recordings of USVs are extensively used in preclinical studies of affect, motivation, and social behavior. Rat USVs are sensitive to the effects of certain classes of psychoactive drugs, suggesting that emission of rat USVs can have relevance not only to neurobiology, but also to neuropharmacology and psychopharmacology. This review summarizes three types of rat USVs, namely 40-kHz USVs emitted by pups, 22-kHz USVs and 50-kHz USVs emitted by young and adult animals, and relevance of these vocalizations to neuropharmacological studies. Attention will be focused on the issues of how rat USVs can be used to evaluate the pharmacological properties of different classes of drugs, and how rat USVs can be combined with other behavioral models used in neuropharmacology. The strengths and limitations of experimental paradigms based on the evaluation of rat USVs will also be discussed.

1. INTRODUCTION

The emission of ultrasonic vocalizations (USVs) is a major means of communication used by rats [1-4]. Three types of rat USVs have been identified so far, which are categorized according to their average peak or dominant frequency, namely the “22-kHz”, the “40-kHz”, and the “50-kHz” USVs [5]. While 40-kHz USVs are emitted by rat pups, 22-kHz and 50-kHz USVs are emitted by young and adult rats [5]. Detailed descriptions of the origin, neurobiological, and pharmacological mechanisms of USV emission by young and adult rats, along with their acoustic features, are provided elsewhere in this issue [6]. Several lines of evidence have demonstrated that rats emit USVs in response to a wide range of stimuli that may produce either euphoric (positive) or dysphoric (negative) states. Rat pups emit 40-kHz USVs, termed also distress calls, when separated from their mother and litter, since they perceive this situation as stressful and threatening [5,7]. In the young (post-weaning rats) and adult rats, stressful and threatening situations initiate emission of 22-kHz USVs [7], whereas appetitive and pleasurable situations promote emission of 50-kHz USVs [2,8,9].

Taken together, these findings envision USVs as a powerful tool in preclinical studies of affect, motivation, and social behavior. Emission of USVs by rats can be also influenced by certain classes of drugs [10-15]. This observation is of paramount interest, as it indicates that measurement of rat USVs can have relevance not only to neurobiological mechanisms, but also to neuropharmacology and psychopharmacology. In line with this, rat USVs are currently being used at the preclinical level to study the effects elicited by different classes of drugs, and to study the neurobiological mechanisms of these effects. USVs appear particularly suitable for neuropharmacological studies, as they have a marked ethological component and are emitted by rats in natural situations. Therefore, the use of USVs can provide straightforward experimental models that usually do not necessitate extensive training on the part of researchers and/or use of other complex procedures (e.g. food or water deprivation), which may affect the intended results.

This review summarizes the potential use of rat USVs in neuropharmacology and drug evaluation, and discusses the strengths and limitations of USVs-based experimental paradigms. Attention will be paid to the different classes of drugs that can be studied by recording and analysis of rat USVs, and to the different experimental models that can be paired with and complemented by evaluation of rat USVs.

2. USE OF RAT 40- kHz ULTRASONIC VOCALIZATIONS IN BEHAVIORAL NEUROPHARMACOLOGY

When separated from their mother and littermates, rat pups emit USVs which comprise broad range of frequencies between 30 and 65 kHz, and are generally referred to as distress calls or 40-kHz USVs, based on their average peak frequency [5]. Rat pups develop the ability to emit these USVs shortly after birth and maintain it until

weaning, at about 18 days of age [16]. In order to understand the behavioral significance of 40-kHz USVs, it has to be considered that newborn rat pups are completely dependent on their mother for surviving, and that they emit 40-kHz USVs specifically in response to isolation from the nest and mother. These observations have suggested that the emission of 40-kHz USVs in response to isolation may be regarded as a direct correlate of distress and/or anxiety in the rat pup [5,7]. This view is also corroborated by the results of other independent studies that have shown how anxiolytic drugs reduce the number of 40-kHz USVs emitted by isolated rat pups. Interestingly, different classes of anxiolytic drugs are able to elicit this effect, including those most commonly used in the clinic such as benzodiazepines and agonists of the serotonin 5-HT_{1A} receptors [17-19] (Table 1). Moreover, the emission of 40-kHz USVs by isolated rat pups can be attenuated by drugs that possess combined anxiolytic and antidepressant properties, such as selective inhibitors of the serotonin transporter (SSRIs) [20] (Table 1). Further support to the hypothesis that emission of 40-kHz USVs may be a juvenile homologue of anxiety in rat pups comes from the evidence that this behavioral response is exacerbated when pups are either administered pentylenetetrazole (PTZ), a drug that possess anxiogenic properties [21], or exposed to low temperatures that act as a stressful stimulus [16]. Taken together, these findings indicate that evaluating the magnitude of 40-kHz USVs emission in isolated rat pups may represent a useful tool for screening the anxiolytic properties of drugs.

Notwithstanding these findings, it has to be pointed out that while measuring 40-kHz USVs in rat pups may provide a reliable experimental model for evaluating benzodiazepine-like drugs and agonists of the serotonin 5-HT_{1A} receptors, the same paradigm may not be suited for screening the anxiolytic properties of other classes of drugs. In this regard, previous studies have shown that certain drugs that have clinically relevant anxiolytic potential (e.g. clonidine, an agonist of norepinephrine α_2 receptors) amplify, instead of attenuating, the emission of 40-kHz USVs by isolated rat pups [22] (Table 1). Therefore, a possible occurrence of false negative results should be carefully considered when utilizing this behavioral paradigm for evaluating the anxiolytic properties of new drugs. Moreover, it is interesting to mention that, although drug screening studies usually focus on the number of vocalizations emitted, drugs can also influence the duration of 40-kHz USVs in isolated rat pups, [10] (Table 1). A recent study that compared the effects of various drugs with anxiolytic properties on emission of 40-kHz USVs has indicated that the duration of these USVs may be more sensitive to the anxiolytic effects of drugs than the number of vocalizations emitted [10]. In addition, the same study has suggested that the measurement of duration of 40-kHz USVs could enable easier discrimination of anxiolytic effects from potential sedative effects of drugs than the number of vocalizations [10]. Therefore, a combined analysis of both the numbers and the duration of 40-kHz USVs emitted by isolated rat pups could help to show anxiolytic effects of drugs that are not adequately revealed by recording the number of vocalizations only.

Besides the data demonstrating that 40-kHz USVs emitted by isolated rat pups are sensitive to the anxiolytic properties of drugs, further experimental findings have been collected that suggest a link between these USVs and anxiety-like states. Previous studies have consistently demonstrated that different neurotransmitters known to participate in the pathophysiology of anxiety, such as γ -aminobutyric acid (GABA), endogenous cannabinoids, and glutamate, can modulate the emission of 40-kHz USVs by isolated rat pups [23,24]. Moreover, experiments performed in the Tsukuba genetic model of low (TLE) and high (THE) emotional rat pups have shown that the emission of 40-kHz USVs stimulated by isolation is significantly more marked in THE pups [25], which also show a more pronounced anxiety-like phenotype at adulthood, compared with TLE pups [26]. Furthermore, a reduced binding of the antagonist flumazenil to the benzodiazepine receptor has been described in the brain of rat pups subjected to isolation, and a similar finding has been observed in patients suffering panic disorders by means of single-photon emission computed tomography (SPECT) studies with the antagonist iomazenil [27,28]. This latter finding has led to the hypothesis that the emission of 40-kHz USVs elicited by maternal separation in rat pups could have face validity towards the separation-induced anxiety that can occur in children [17], although this has to be conclusively demonstrated. Taken together, these findings indicate that 40-kHz USVs emitted by isolated rat pups could serve as a model suited not only for the screening of anxiolytic drugs, but also for studying the neurobiological mechanisms of anxiety, the individual traits, and the neurodevelopmental factors that may promote the manifestation and influence the severity of this pathology.

In addition to being suited for the study of anxiety-like states and their pharmacological treatment, 40-kHz USVs emitted by isolated rat pups may be of interest for the investigation of all those conditions, either pharmacological or pathological, that may harm the fetus and the newborn during the prenatal and perinatal phases of development. This experimental use of 40-kHz USVs is supported by evidence showing that the ability of rat pups to vocalize in a species-typical manner when isolated from the nest is dependent on the integrity of the central nervous system (CNS) (see [29] for a discussion). Therefore, modifications of this behavioral response can be assumed as indicative of neurological impairments in the affected pups. Furthermore, the use of 40-kHz USVs triggered by isolation allows to study not only those insults that may directly target the pup after birth (e.g., perinatal asphyxia and hypoxia), but also those factors (e.g., infections) and maternal behaviors (e.g., substance abuse) that can harm the fetus during gestation. Modifications in the number of 40-kHz USVs emitted and their acoustic features have been reported in isolated rat pups previously exposed to diverse neurological insults, such as febrile convulsions, global asphyxia, perinatal hypoxic-ischemic encephalopathy, and gestational exposure to lipopolysaccharide [30-33]. Interesting findings have also been obtained in rat pups delivered by dams allowed to drink ethanol during gestation. The pups exposed to ethanol have been reported to emit lower numbers of 40-kHz USVs following isolation, compared to non-exposed pups

[34]. Also, changes in vocalization in ethanol-exposed pups were found to be associated with other behavioral impairments, such as loss of righting reflex, reminiscent of some of the symptoms observed in infants suffering from fetal alcoholic syndrome (FAS) [35]. Similar deficits in the emission of 40-kHz USVs following isolation have been reported in rat pups delivered by dams treated with cocaine during pregnancy [29,35].

Moreover, it is worth mentioning the results of other studies which have demonstrated that drugs of abuse that may be toxic for the brain impair the emission of 40-kHz USVs by isolated rat pups when administered in the post-natal period. Such results have been reported for ethanol, 3,4-methylenedioxymethamphetamine (MDMA, or “ecstasy”), and morphine [35-37]. Besides the relevance to pharmacological and toxicological insults, it is noteworthy that the analysis of 40-kHz USVs emitted by isolated rat pups in different situations may also be used for investigating the pups’ ability to communicate with their mother, which is a critical process in neurobehavioral development. In this regard, it is worth mentioning that an increased emission of 40-kHz USVs has been observed in isolated rat pups subject to prenatal stress [38]. Moreover, a decreased emission of these USVs has been reported in rats that underwent early maternal separation [39]. Interestingly both these experimental models reproduce factors that can critically influence the proper development of social and communicative functions [40,41].

The findings discussed so far suggest that parameters of 40-kHz USVs emitted by rat pups in response to isolation may be regarded as a behavioral correlate of neurological and neuropsychiatric abnormalities. However, other lines of evidence suggest that the emission of 40-kHz USVs by isolated rats pups may be critically influenced by the functioning of the cardiovascular system, and that a decrease in venous return may trigger these USVs [42]. As mentioned earlier, the emission of 40-kHz USVs by isolated rat pups can be powerfully stimulated by exposure to cold, which is associated with an increase in blood viscosity and a reduction in heart rate [43]. Moreover, the administration of antihypertensive drugs, such as the above mentioned clonidine, chlorisondamine (a ganglionic blocker that elicits vasodilation and bradycardia), and sodium nitroprusside, (a directly acting dilator of arteries and veins) has been demonstrated to stimulate the emission of 40-kHz USVs by isolated rat pups [42] (Table 1). The possible influence of cardiovascular function on the emission of 40-kHz USVs deserves consideration. On the one hand, this might contribute to explanation of the false negative results that may be observed in this paradigm after the administration of anxiolytic drugs (see above). In line with this, it can be hypothesized that the effects elicited by clonidine on 40-kHz USVs in isolated rat pups could stem from the antihypertensive, rather than anxiolytic, properties of the drug [42]. On the other hand, the emission of 40-kHz USVs by isolated rat pups could be envisioned as a relevant behavioral parameter to be used in the investigation of cardiovascular development and toxicity [43]. However, this has not specifically been

addressed yet, and exhaustive experimental studies need to be performed to corroborate this proposed use of 40-kHz USVs.

Taken together, the available experimental evidence indicates that the emission of 40-kHz USVs by isolated rat pups may represent a valid experimental tool for both the screening of new drugs, in particular anxiolytic drugs, and the investigation of the factors that can harm the newborn and influence its development. Although the concept that rat pup 40-kHz USVs may be a straightforward correlate of anxiety has been questioned [7,42], and some limitations, such as the occurrence of false negative results [22,42], may complicate the use of rat 40-kHz USVs for drug evaluation, they still represent a significant measure of developing anxiety in rat pups.

3.USE OF RAT 22- kHz ULTRASONIC VOCALIZATIONS IN BEHAVIORAL NEUROPHARMACOLOGY

Young and adult rats emit the so-called 22-kHz USVs in response to a wide series of stimuli they perceive as threatening; therefore 22-kHz USVs are often referred to as “alarm calls” [2,4,7]. These vocalizations are characterized by a relatively constant frequency and a long duration (up to seconds). It needs to be mentioned that the duration of 22-kHz USVs is significantly longer than that of both 40-kHz and 50-kHz USVs, that are generally of 20-150 ms in duration [2,6]. Rats can also emit 22-kHz USVs with a shorter duration. With regard to these latter USVs, it is interesting to mention that a recent study has demonstrated that rats that self-administer binges of cocaine emit short 22-kHz USVs [44]. Furthermore, earlier investigations have observed the emission of short 22-kHz USVs in naïve rats subjected to handling and in rats exposed to foot shocks [45,46]. While these latter findings seem to suggest that short 22-kHz USVs may be produced in response to aversive stimuli, the precise behavioral significance of the USVs is still unclear [2], and studies that evaluate the emission of 22-kHz USVs usually rely on those vocalizations that have a long duration (see [6] for a discussion on the possible communicative function of the short 22 kHz USVs). The use of 22-kHz USVs in neuropharmacology and drug screening chiefly involves the study of anxiolytic and mixed anxiolytic/antidepressant drugs. Moreover, the emission of 22-kHz USVs can be evaluated, together with rats’ performance in other behavioral paradigms, in investigations aimed at elucidating the mechanisms and neuronal circuits that are involved in neuropsychiatric disorders (e.g. anxiety, depression).

Different types of experimentally-delivered stressful stimuli, such as air puffs, electric foot-shocks, and loud noises, can stimulate the emission of 22-kHz USVs, and these stress-induced vocalizations have been proposed to model an anxiety-like state in the rat [47]. In general, the use of 22-kHz USVs for the screening of anxiolytic drugs relies on conditioning paradigms, in which rats are expected to vocalize in anticipation of a stressful stimulus, usually an electric foot-shock, to which they have previously been exposed [47-50]. Although different experimental protocols

exist, which may differ with regard to the apparatus used and the duration of conditioning, the procedure to attain the conditioned emission of 22-kHz USVs by rats is generally performed as follows. Rats are firstly individually placed in a soundproof chamber and randomly exposed to a train of unavoidable foot-shocks [47-49]. To facilitate conditioning, in some variants of this procedure, foot-shocks are paired with other contingent stimuli, either visual or acoustic [47-49]. Conditioning to foot-shocks is then repeated until the test day, when rats are exposed to the same chamber but no foot-shocks are delivered. In the course of this experimental paradigm, rats will progressively develop environmental conditioning, and will emit anticipatory 22-kHz USVs when re-exposed to the environment where unavoidable foot-shocks were previously delivered. This behavioral response is based on the fact that rats will perceive this situation as threatening since it has been previously associated with the presentation of unavoidable stressful stimuli [47-49]. When this procedure is applied to drug screening, the endpoint is to evaluate whether drugs attenuate or suppress the emission of 22-kHz USVs by rats conditioned to this situation [47].

Other studies have demonstrated that drugs with clinically relevant anxiolytic properties, such as the benzodiazepine diazepam [51] and the 5HT_{1A} receptor agonists buspirone and gepirone [51,52], effectively attenuate the emission of 22-kHz USVs by rats conditioned to stress (Table 2). Further support to a possible link between stress-induced 22-kHz USVs and anxiety comes from studies showing that the emission of these vocalizations is usually associated with behaviors that are thought to indicate an anxiety-like state in rats (e.g. freezing, decreased rearing) [47], and is exacerbated by the administration of the anxiogenic drug PTZ [51]. In line with this finding, it is also noteworthy that some studies have demonstrated that drugs that attenuate stress-induced 22-kHz USVs are often also able to counteract anxiety-like behaviors in well-standardized behavioral paradigms, such as the elevated-plus maze (EPM) or the Vogel conflict test [53-55]. Taken together, these observations indicate that the emission of stress-induced 22-kHz USVs can be considered an appropriate experimental model to be used in the evaluation of the anxiolytic properties of drugs. Nevertheless, this paradigm has some limitations which have to be carefully considered in order to properly interpret the results obtained.

Studies with benzodiazepines have demonstrated that while diazepam significantly attenuates the emission of stress-induced 22-kHz USVs by rats, other drugs in this class (e.g. chlordiazepoxide, zolpidem) are significantly less effective [47,50,56]. Moreover, diazepam has often been reported to act on stress-induced 22-kHz USVs when administered at doses close to those at which the drug elicits sedative effects [47]. Taken together, these findings indicate that the effects of benzodiazepines on the emission of stress-induced 22-kHz USVs by rats may vary with the specific drug evaluated. In addition, they suggest that complementary tests to evaluate the presence of sedation should be performed when benzodiazepine-like drugs are screened by measuring their effects on stress-induced 22-kHz USVs.

Additional pharmacological studies have suggested that the emission of 22-kHz USVs by rats conditioned to stress could also mimic panic-like anxiety, and that this paradigm may represent a particularly useful tool for testing drugs designed for panic-like disorders. A significant attenuation of the emission of stress-induced 22-kHz USVs has been reported in rats that were administered either with SSRIs, such as citalopram, fluoxetine, paroxetine [57], or with the benzodiazepine alprazolam [50], all of which have specific indication for the treatment of panic-like anxiety [58]. In line with what discussed above, the efficacy of alprazolam has been found to be higher than that of other benzodiazepines [50], which would further corroborate the proposed link between stress-induced 22-kHz USVs and panic-like anxiety.

The vast majority of drug screening studies that have investigated the emission of 22-kHz USVs by rats conditioned to stress have employed acute administration protocols. Few studies have been performed so far that have evaluated the effects of chronic drug administration on stress-induced 22-kHz USVs, and these studies have yielded mixed results. On the one hand, rats chronically administered with benzodiazepine-like drugs have been reported not to develop tolerance to the drug-induced decrease in the emission of stress-induced 22-kHz USVs [59,60]. On the other, the emergence of tolerance to this effect has been described in rats conditioned to stress and chronically treated with novel anxiolytic agents, such as agonists of the neurotensin NTS1 receptor [61]. Therefore, while the attenuation of stress-induced 22-kHz USVs stands as a valid and widely used paradigm for the evaluation of the anxiolytic properties of drugs, all the above described limitations should be carefully considered. In particular, the thorough elucidation of the chronic effects of drugs on these vocalizations appears particularly relevant to the development of new anxiolytics, as the clinical treatment of anxiety and its related disorders may often require drugs that are able to maintain their effects after a long-term treatment.

In addition to the use in the screening of anxiolytic drugs, stress-induced 22-kHz USVs may be of interest for the investigation of the neurobiological bases of emotionality, as well as for the characterization of the pharmacological and pathological factors that may influence the emotional state of rats. Evaluating the emission of stress-induced 22-kHz USVs has been a useful tool in neuroanatomical and neuropharmacological studies that have contributed to characterization of brain circuits engaged in the manifestation of anxiety-like behaviors in rats. This approach has demonstrated that various neurotransmitter systems (e.g., GABA, norepinephrine, serotonin, neurokinin-1, neurotensin) and brain regions (e.g., forebrain cholinergic nuclei, periaqueductal gray, medial prefrontal cortex) participate in these behaviors [2,47,48,51,62]. A sustained emission of stress-induced 22-kHz USVs has also been reported in rats subjected to withdrawal from different drugs, including diazepam [63,64], and substances of abuse such as cocaine, ethanol, or opiates [65-68]. These findings indicate that stress-induced 22-kHz USVs may be a valid tool for investigating the

anxiety-like states associated with drug withdrawal, and for elucidating the role these states may have in drug relapse and establishment of drug dependence.

Finally, the emission of stress-induced 22-kHz USVs has been used in longitudinal studies aimed at investigating the influence of prenatal and perinatal insults on emotionality in later life, with particular attention to the development of anxiety- and depressive-like phenotypes. These studies have yielded interesting results which show how rats exposed to stressful events in early life, such as maternal separation, maternal immune activation reminiscent of perinatal infections, or isolation rearing, display significant changes in the emission of stress-stimulated 22-kHz USVs in the adulthood, revealed by either an increase or a decrease in the number of vocalizations compared with non-exposed rats [69-72]. It is important to note that in the case of maternally-separated rats, changes in the emission of stress-induced 22-kHz USVs have been found to positively correlate with the presence of a depressive-like phenotype, measured as an increase in immobility time in the forced swimming test [69].

Besides the study of neuropsychiatric diseases and their treatment, 22-kHz USVs can also be used as a complementary behavioral parameter in experimental models of pain. In general, in these models, the emission of 22-kHz USVs can either be spontaneous or elicited by the application of mechanical stimuli. The magnitude of the vocalization, measured either as the number of 22-kHz USVs emitted per time unit, or the total number of seconds of vocalization, has been regarded as a direct correlate of the nociceptive response [73,74]. Independent studies have demonstrated a sustained emission of 22-kHz USVs by rats subjected to different experimental models of pain, such as electric stimulation of the tail, formalin-induced paw inflammation, Freund's adjuvant-induced arthritis, intramuscular injection of cephalosporins, and paw incision model of postoperative pain [73-76]. Moreover, it has been demonstrated that the emission of 22-kHz USVs by rats subjected to experimental chronic pain can be attenuated by the administration of drugs that possess clinically relevant analgesic properties, such as morphine and non-steroidal anti-inflammatory drugs (NSAIDs) [13,74,75]. Additional studies have shown that novel drugs and natural substances with proposed analgesic activity also are able to modulate the emission of 22-kHz USVs associated with experimental chronic pain in the rat [76,77]. Taken together, these findings envision the evaluation of 22-kHz USVs as a potentially useful tool for the development of novel analgesic therapies. However, it has to be acknowledged that the existence of a link between the emission of 22-kHz USVs and pain states in the rat is still disputed, and some of the results obtained by previous investigations on this issue require further consideration.

Some of the studies that have demonstrated a sustained emission of USVs in rat models of pain have failed to unambiguously identify the 22-kHz USVs of long duration as the vocalization subtype specifically emitted under the experimental circumstances evaluated. In fact, the emission of either 22-kHz USVs of short duration [74] or USVs with

a frequency contained between 60 and 80-kHz [73] has also been recorded in experimental models of pain. While this finding could be an artifact of either the experimental procedure or the USVs recording equipment used, if clearly demonstrated, it would challenge the idea that 22-kHz USVs of long duration are “the” USVs subfamily whose emission is specifically associated with pain states.

In addition, studies that have evaluated the emission of 22-kHz USVs by rats subjected to experimental pain have yielded mixed results. Experiments performed in rat models of arthritis have shown that procedures such as the extension or the compression of the knee can stimulate the emission of 22-kHz USVs [78,79]. Conversely, other studies failed to observe any significant emission of 22-kHz USVs by rats subject to Freund’s adjuvant-induced arthritis [80]. Furthermore, a detailed study that has compared the emission of 22-kHz USVs across experimental rat models of inflammatory pain (formalin test), neuropathic pain (partial sciatic nerve ligation), and visceral pain (referred hyperalgesia), has found no positive correlation between the emission of 22-kHz USVs and other behaviors that are indicative of pain in these models, such as licking and hind paw withdrawal [81]. However, as mentioned earlier in this review, others have reported a sustained emission of 22-kHz USVs by rats subject to these and others models of chronic pain [73-79]. In considering these results together, it is important to remark that the studies on 22-kHz USVs and pain described here often differed with regard to the experimental procedures used. Experiments performed in rat models of arthritis have evaluated either the spontaneous emission of 22-kHz USVs, or the vocalization in response to the application of mechanical stimuli [13,77-80]. In a similar fashion, studies with the formalin test have evaluated behavioral responses and 22-kHz USVs emission by using different concentrations of the irritant [74, 81].

While experimental discrepancies could have certainly contributed to the mixed results reported in the literature, it is still controversial whether the emission of 22-kHz USVs could be considered a parameter that directly relates to the intensity of nociception in rats. Nevertheless, 22-kHz USVs are currently widely used as an additional behavioral measure in the study of experimental chronic pain and, in this regard, it is noteworthy that recent findings have suggested that these vocalizations could model not only nociception, but also the affective component of pain [74]. Pain involves both a sensory component, which depends on the transmission of nociceptive stimuli, and an affective component, which consists of the desire to attenuate the intensity of pain, and eventually terminate it [82]. In consideration of the above discussed relationship between 22-kHz USVs and anxiety-like states, it could be possible that these vocalizations may represent an index of distress or anxiety associated with pain. This interpretation could contribute to explaining the inconsistent results obtained in studies that have evaluated the emission of 22-kHz USVs in rats subjected to experimental pain. It is conceivable that the intensity of nociception would not necessarily match the level of distress associated with pain. Furthermore, the relevance of 22-kHz USVs to the affective component of pain is

also indirectly substantiated by the finding that morphine can attenuate the emission of 22-kHz USVs in the rat models of pain [74], because morphine exerts its analgesic effects not only by acting at the level of the nociceptive pathways, but also by improving the affective perception of pain [83,84].

In addition to their use in the screening of anxiolytics and analgesics, rat 22-kHz USVs could also be a useful tool in the development of other classes of drugs. Stress-induced 22-kHz USVs can be used, in combination with other behavioral measures, for evaluating the pharmacological profile of antipsychotic drugs [85], based on the different ability of so called “classical” (e.g., haloperidol) and “atypical” (e.g., clozapine) antipsychotics to attenuate conditioned responses [85,86]. The evaluation of the effects of antipsychotics on stress-induced 22-kHz USVs can also disclose the presence of additional anxiolytic properties of these drugs [85]. Moreover, 22-kHz USVs could have some relevance to cardiovascular pharmacology. Previous experiments have demonstrated that the emission of 22-kHz USVs that occurs during conditioned fear is associated with changes in peripheral physiological parameters such as increase in blood pressure and heart rate [87]. Moreover modifications in the acoustic parameters, but not numbers, of 22-kHz USVs have been shown to positively correlate with blood pressure and heart rate [88]. (See [6] for emotional expression containing concurrent emission of vocalization and manifestation of emotional autonomic symptoms). Taken together, these findings would suggest that the evaluation of the acoustic features of stress-induced 22-kHz USVs could represent a complementary, non-invasive, method for the performance of cardiopharmacology experiments in rats. The effects of various drugs on the emission of 22-kHz USVs in different experimental models are summarized in Table 2.

4.USE OF 50-kHz ULTRASONIC VOCALIZATIONS IN BEHAVIORAL NEUROPHARMACOLOGY

Fifty-kHz USVs are characterized by a high peak frequency, usually contained within 35–80 kHz, and a short duration, usually within 10–150 ms [2]. Young and adult rats emit these USVs in response to, or anticipation of, pleasurable stimuli such as mating, heterospecific play, namely the procedure known as “tickling” by a human hand that follows rough-and-tumble play of juveniles, and non-aggressive social encounters with conspecifics [2,8,9]. These lines of evidence have suggested that the emission of 50-kHz USVs may represent a behavioral parameter that indicates “positive” affect in the rat. In line with this, 50-kHz USVs are increasingly being used in experiments for investigating those situations and factors that may promote the manifestation of positive affective states [2-4,9]. Interestingly, previous pharmacological studies have demonstrated that the emission of 50-kHz USVs can be modulated by certain drugs that possess rewarding and addictive properties. A sustained emission of 50-kHz USVs has been observed after the administration of the dopaminergic psychostimulant amphetamine [15,89]. Similar findings have been reported in rats subject to environmental conditioning to amphetamine or morphine [90]. These results have been later replicated, and confirmed by showing that other substances of abuse, such as cocaine, MDMA, and nicotine, as well as the

psychostimulant caffeine, may influence the emission of 50-kHz USVs in rats [11,14,91-94]. Taken together, these findings would suggest that 50-kHz USVs may represent a behavioral response that can be measured in studies concerned with the motivational effects of drugs of abuse, either alone or in combination with other paradigms such as conditioned place preference (CPP) [95], and drug self-administration [44,96,97]. The evaluation of 50-kHz USVs during self-administration appears a promising strategy that may disclose the emotional components inherent to drug abuse, and the factors that may promote relapse and reinstatement of drug taking. A detailed discussion of the usefulness and relevance of 50-kHz USVs to drug self-administration is provided elsewhere in this issue [98].

Studying the motivational properties of drugs by means of 50-kHz USVs may nevertheless have some limitations that need to be carefully considered. In this regard, it is noteworthy that evidence has been accumulated, which demonstrates that the effects of drugs of abuse on the emission of 50-kHz USVs by rats may significantly differ. On the one hand, many independent studies have consistently demonstrated that the dopaminergic psychostimulants amphetamine and cocaine elicit the emission of a high number of 50-kHz USVs immediately after their administration to rats [11,15,94]. On the other hand, MDMA, morphine, and nicotine, which also possess rewarding and addictive properties have been reported not to elicit this effect, even after their repeated administration [91,92]. Previous results obtained in rats which engaged in playful social contacts have suggested that the number of vocalizations emitted may code the affective significance of 50-kHz USVs [99]. Based on this, the results summarized above would suggest that the number of 50-kHz USVs emitted immediately after drug administration should not be considered a straightforward correlate of the motivational properties of drugs, at least in the case of non-dopaminergic drugs. It is still unclear why certain drugs that have rewarding properties fail to stimulate the emission of 50-kHz USVs immediately after their administration. One possible explanation of this could rely on the mechanism(s) by which rewarding drugs elicit their central effects, and on the crucial influence of dopamine transmission in the nucleus accumbens (NAcc) shell on the emission of 50-kHz USVs [100,101]. While a thorough discussion of how different rewarding drugs act at the molecular level goes beyond the aims of the present review, it has to be mentioned that dopaminergic psychostimulants promote a massive release of dopamine in the NAcc shell [102], but other rewarding drugs elicit this effect to a lower extent [103]. Besides, experimental evidence also exists showing that norepinephrine and glutamate may modulate the emission of 50-kHz USVs [104,105]. Based on these considerations, it is probable that the effects of drugs with rewarding properties that elicit emission of 50-kHz USVs recorded immediately after their administration may differ in their action from the general effects these substances have on both dopamine and non-dopaminergic neurotransmitters.

Interestingly, drugs that fail to stimulate a sustained emission of 50-kHz USVs by rats may nevertheless influence other features of these vocalizations. Previous experiments have shown that MDMA, morphine, and nicotine

can modify the acoustic features (peak frequency and bandwidth) of the 50-kHz USVs that are recorded immediately after their administration [14] (Figure 1). Similar findings have also been reported for the psychostimulant caffeine [93]. It has been suggested that the maximum peak frequency, together with the number of vocalizations emitted, could code the affective significance of 50-kHz USVs [99]. Moreover, different subtypes of 50-kHz USVs have been isolated, and it has been proposed that each of them could have a different behavioral significance in terms of rats' affective state [106]. (For current characteristics and classification of 50 kHz calls and their subtypes see [6]). Interestingly, MDMA, morphine, and nicotine, while failing to elevate the total number of 50-kHz USVs emitted by rats, have been shown to modify the number of certain subtypes of these vocalizations [14] (Table 3). Notwithstanding these considerations, no convincing information is currently available that links the acoustic features of drug-stimulated 50-kHz USVs to the motivational properties of drugs. Similarly, no sufficient evidence has been collected so far to conclude that drug-induced changes in the relative numeric proportion of the various 50-kHz USVs subtypes may reflect different aspects of the motivational properties of drugs. In this regard, it is worth mentioning that previous studies have suggested that the “trill” subtype of 50-kHz USVs, may be a selective indicator of drug-induced positive affect, since a sustained emission of this USVs subtype has been reported following amphetamine administration [8,106,107]. However, more recent investigations that have examined the effects of amphetamine, methamphetamine, and morphine on the different subtypes of 50-kHz USVs have led to mixed results, and showed either a decrease or no changes in the number of “trill” vocalizations after administration of these drugs [92,95,108]. On these bases, additional studies are needed to clarify whether modifications in the acoustic features and subtypes of drug-induced 50-kHz USVs may somehow relate to the motivational properties of rewarding drugs.

Although the precise significance of the 50-kHz USVs emitted by rats immediately after drug administration may need further discussion, clearer data have been obtained in experiments that have examined the long-term effects of drugs on 50-kHz USVs. As mentioned earlier, previous studies have demonstrated that rats treated with either amphetamine, MDMA, morphine or nicotine in a novel environment exhibit a sustained emission of 50-kHz USVs when later re-exposed to the environment where the drug was administered [90,92]. Similar findings have also been observed in rats previously trained to self-administered cocaine [109]. Taken together, and considering that 50-kHz USVs are emitted in anticipation of pleasurable stimuli, these effects are likely to reflect environmental drug conditioning and, in turn, to indicate the desire for drug by previously drug-experienced rats. Moreover, a comparative study of the long-lasting changes in 50-kHz USVs emitted by rats subjected to drug withdrawal and re-exposed to a previously drug-paired environment has demonstrated that this response was significantly more marked and persistent in morphine-treated rats than in rats treated with amphetamine, MDMA, or nicotine [92]. These results would be in line

with the ability of opiates to induce intense craving, which can persist long after drug discontinuation [110,111]. Therefore, these findings would suggest a possible use of conditioned 50-kHz USVs as a tool for investigating long-term effects of drugs of abuse and craving associated with drug withdrawal. Taken together, the results obtained with conditioned 50-kHz USVs would suggest that these vocalizations could be a more reliable indicator of the motivational properties of drugs of abuse than the 50-kHz USVs recorded immediately after drug administration.

Besides being used for evaluation of drug effects in relation to dependence, the emission of 50-kHz USVs could be a relevant behavioral parameter to be included in longitudinal studies that investigate the effects of early drug exposure on later changes in emotionality. In this regard, it is interesting to mention that a wealth of studies performed in different species of experimental animals have demonstrated a positive association between the emergence of delayed cognitive development, emotional abnormalities and the exposure in early life to drugs of abuse, such as cocaine, ethanol, and Δ^9 -tetrahydrocannabinol (THC) [112-114]. These considerations also apply to the human setting, as similar findings have been obtained in clinical and epidemiological studies [115-116]. In this regard, it is also worth mentioning that the exposure to drugs of abuse often begins as early as during gestation, in the case of children born from dependent mothers, or at adolescence [117-118], both of which are critical phases in brain development [119]. As of today, the only studies available concerned with the long-term effects of early drug exposure on the emission of 50-kHz USVs at later life stages have investigated how prenatal ethanol exposure may affect the ability of rats to vocalize in response to behavioral challenges at adolescence or adulthood. However, these studies have obtained mixed results. On the one hand, prenatal exposure to ethanol has been reported not to affect the emission of 50-kHz USVs by adult rats during copulatory behavior [120]. On the other hand, an increased emission of both 50-kHz and 22-kHz USV in response to a mild stress has been reported in adolescent rats exposed to ethanol during the prenatal period, compared with non-exposed rats [121]. It is possible that methodological issues such as the protocol of ethanol administration, or the specific stimulus used to elicit USVs could underlie these discrepancies. Nevertheless, in light of the behavioral significance of 50-kHz USVs discussed elsewhere in this review, the evaluation of 50-kHz USVs appears a potentially relevant behavioral measure to be included in longitudinal studies that assess the long-term neurobehavioral effects caused by the exposure to drugs of abuse in early life.

While the study of drugs of abuse is currently one of the fields where rat 50-kHz USVs are most extensively used, experimental evidence suggests that these vocalizations may be relevant also to other aspects of neuropharmacology. In this regard, interesting results have been obtained by studies that have examined the emission of 50-kHz USVs stimulated by tickling. Briefly, tickling consists of manipulation of rats according to a specific protocol in order to reproduce the features of the rough-and-tumble play that occurs among juvenile rats [8,122,123]. Previous studies have

observed that rats differ in regard to number of 50-kHz USVs emitted in response to tickling and that, accordingly, they can be classified in two populations, one displaying a high and the other a low emission of 50-kHz USVs [124]. Rats that display the low emission of 50-kHz USVs in response to tickling present both a phenotype and a genotype that are reminiscent of those featuring autism in humans, when compared with rats that respond to tickling by emitting the high number of 50-kHz USVs [125,126]. In fact, the former rats have been shown to engage in less social contacts with their conspecifics, and to show a differential expression of the gene encoding for the glutamate N-methyl-D-aspartate (NMDA) receptor, the altered function of which has been proposed to be critically involved in the pathophysiology of autism [127]. Pharmacological experiments performed in this putative model of autism have demonstrated that GLYX-13, a drug acting as a partial agonist of the glycine site of the NMDA receptor, effectively counteracts the deficits in 50-kHz USVs emission observed following tickling [127]. GLYX-13 is currently under clinical investigation [128,129], which lends support to the idea that 50-kHz USVs may represent a useful behavioral measure in the screening and preclinical development of new drugs to be used in the treatment of neuropsychiatric diseases.

Interesting results on tickling-induced 50-kHz USVs have also been obtained in a recent study that has suggested that deficits in the emission of these vocalizations could be a behavioral parameter indicative of negative symptoms in pharmacological models of schizophrenia [130]. In line with previous findings [131], this study found that the administration of phencyclidine and MK-801 (also known as dizocilpine), two NMDA receptor antagonists that are widely used to induce schizophrenia-like phenotypes in rats [132], attenuates the emission of 50-kHz USVs in rats subjected to tickling [130]. The same study observed that buspirone, which has a clinically relevant potential in counteracting the negative symptoms of schizophrenia, effectively reversed the deficit in tickling-induced 50-kHz USVs [130]. This finding is of great interest, as it would substantiate the relevance of 50-kHz USVs for the experimental modeling of the negative symptoms of schizophrenia. However, it has to be mentioned that aripiprazole, which also has clinical indication for the treatment of negative symptoms of schizophrenia, had no beneficial effects in the same rat model [130]. Therefore, further studies should be performed to ascertain whether deficits in 50-kHz USVs may be considered a good behavioral correlate of experimental psychosis, and which of the negative symptoms of schizophrenia can be specifically modeled by changes in tickling-induced 50-kHz USVs.

Another potential use of tickling-induced 50-kHz USVs could be as a tool for evaluating the neurobiological mechanisms of anxiety and depression and, in turn, the anxiolytic and antidepressant properties of drugs. This is suggested by previous experiments which have demonstrated that the number of 50-kHz USVs emitted in response to tickling may predict the behavior of rats in tests that evaluate anxiety- and depression-like behaviors, such as EPM, fear conditioning, forced swimming test, and sucrose preference [133]. However, up to now no studies have been performed

that have specifically addressed a possible link between the emission of tickling-induced 50-kHz USVs and the effects of anxiolytic or antidepressant drugs. Nevertheless, additional experimental evidence exists which supports the relevance of 50-kHz USVs to the evaluation of the anxiolytic properties of drugs. As a previous study has demonstrated, rats subjected to social isolation exhibited a sustained emission of 50-kHz USVs when exposed to a conspecific, and the magnitude of this vocalization was higher than that recorded from non-isolated rats [134]. Also, diazepam has been reported to further amplify the emission of 50-kHz USVs in this experimental model [134]. This result suggests that the emission of 50-kHz USVs by socially-isolated rats may be sensitive to the effects of anxiolytic drugs, at least in the case of benzodiazepines, and could potentially be of relevance to the screening of these drugs. Another recent study has suggested that the emission of 50-kHz USVs could be an index of positive affect in experimental mania [135], by showing that 50-kHz USVs stimulated by amphetamine, whose administration can induce mania-like states in rodents [136], could be reversed by lithium and tamoxifen, both of which possess a clinically relevant antimanic potential [137,138]. The proposed relationship between 50-kHz USVs and mania is thoroughly discussed elsewhere in this issue [139]. These results appear very interesting, and suggest that evaluating the emission of 50-kHz USVs could provide a simple paradigm to study mania at the preclinical level, which could in turn enable significant progress beyond the current state of knowledge, since the available experimental models of mania are largely unsatisfactory. Finally, it is noteworthy that, as mentioned above, rats emit 50-kHz USVs during copulatory behavior [140-142]. This has allowed, in the first place, to use the emission of 50-kHz USVs in studies that have investigated neurobiological mechanisms of sexual behavior and its affective components. Moreover, the emission of 50-kHz USVs during copulatory behavior has recently been exploited as a tool to investigate the occurrence of changes in phonation that may accompany neurodegeneration in experimental models of Parkinson disease [143,144]. This latter use of 50-kHz USVs is thoroughly discussed elsewhere in this issue [145].

To summarize, the experimental evidence discussed above suggests that 50-kHz USVs could be of potential relevance to the study of both the neurobiology of different neuropsychiatric disorders and the screening of new drugs to be used in the treatment of these disorders. Moreover, while the emission of 50-kHz USVs is chiefly dependent on brain emotive mechanisms rather than peripheral mechanisms [101,123,146], further studies should be performed to find out whether measurements of 50-kHz USVs could as well have some experimental use for studying processes outside of the central nervous system.

5.CONCLUSIONS

Several lines of experimental evidence have accumulated to suggest that recording and analysis of USVs emitted by rat pups or by young/adult rats could represent a useful tool in neuropharmacology, which may also have potential

implications for other areas of pharmacology. The evaluation of rat USVs can be used either as a self-standing technique, or be combined with other validated models in behavioral pharmacology that are employed to reproduce the features of neurological and psychiatric disorders. Importantly, the emission of USVs is sensitive to the effects of different classes of drugs, hence providing a potential tool to be used in drug screening studies. Nevertheless, the use of rat USVs as an experimental model in neuropharmacology has some intrinsic limitations, which have to be carefully considered. Some of the aspects related to the behavioral significance of rat USVs and their translation to human communication and expression of affect are still to be elucidated. Moreover, the effect of drugs on the emission of USVs may differ from those the same drugs elicit in other behavioral paradigms. Finally, certain drugs that possess clinically relevant effects may have a negligible or unexpected influence on the emission of USVs. Notwithstanding these limitations, rat USVs stand as a new promising tool in neuropharmacology, particularly in light of their marked ethological component, and they create a possibility of combining them with other well-standardized behavioral paradigms, without the need for extensive training and complex animal manipulations.

CONFLICT OF INTEREST

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LEGENDS FOR FIGURES AND TABLES

Figure 1 Effects of different drugs of abuse on the acoustic features of 50-kHz USVs emitted by rats immediately after drug administration. Black columns indicate a significant difference, compared with acute administration of vehicle. Raw numeric data can be found in [14]. AMPH = amphetamine; MDMA = 3,4-methylenedioxymethamphetamine; MOR = morphine; NIC = nicotine. Doses are expressed in mg/kg.

Table 1 Overview of the effects of drugs from different pharmacological classes on the emission of 40-kHz USVs by isolated rat pups. ↑ indicates an increase, ↓ indicates a decrease, and – indicates no changes. For further details please refer to the references quoted in the table.

Table 2 Overview of the effects of drugs from different pharmacological classes on the emission of 22-kHz USVs by rats recorded in different experimental models. ↑ indicates an increase, ↓ indicates a decrease, and – indicates no changes. For further details please refer to the references quoted in the table.

Table 3 Effects of different drugs of abuse on the subtypes of 50-kHz USVs emitted by rats immediately after drug administration. ↑ indicates an increase, ↓ indicates a decrease, and – indicates no changes, all compared with acute administration of vehicle. * indicates a significant difference, compared with acute administration of vehicle. Raw numeric data can be found in [14]. Vocalization subtypes are defined according to [106], and a detailed graphical representation of vocalizations subtypes is provided therein. AMPH = amphetamine; MDMA = 3,4-methylenedioxymethamphetamine; MOR = morphine; NIC = nicotine. Doses are expressed in mg/kg.

Drug	Pharmacological Class	Effect observed
alpidem	imidazopyridine; anxiolytic	↓ number of USVs [17]
alprazolam	benzodiazepine; anxiolytic	↓ number of USVs [17]
amitriptyline	tricyclic; antidepressant	↓ number of USVs [10] ↓ duration of USVs [10]
bretazenil	imidazopyrrolbenzodiazepine; anxiolytic,	↓ number of USVs [17]
bupirone	azapirone, 5HT _{1A} receptor agonist; anxiolytic	↓ number of USVs [10,19] ↓ duration of USVs [10]
chlordiazepoxide	benzodiazepine; anxiolytic	↓ number of USVs [10,17] ↓ duration of USVs [10]
chlorimipramine	tricyclic; antidepressant	↓ number of USVs [20]
chlorisondamine	nicotinic receptor antagonist; ganglionic blocker	↑ number of USVs [42]
citalopram	SSRI; antidepressant with anxiolytic properties	↓ number of USVs [10,20] ↓ duration of USVs [10]
clonidine	central α 2 receptor agonist; antihypertensive with anxiolytic properties	↑ number of USVs [22]
desipramine	tricyclic; antidepressant	↑ number of USVs [20]
diazepam	benzodiazepine; anxiolytic	↓ number of USVs [10,17,18] ↓ duration of USVs [10]
fluoxetine	SSRI; antidepressant with anxiolytic properties	↓ number of USVs [10] ↓ duration of USVs [10]
hydralazine	selective dilator of arteries and arterioles	– duration of USVs [42]
imipramine	tricyclic; antidepressant	– number of USVs [10,20] – duration of USVs [10]
oxazepam	benzodiazepine; anxiolytic	↓ number of USVs [17]
paroxetine	SSRI; antidepressant with anxiolytic properties	↓ number of USVs [20]
ritanserin	5HT _{2A-C} receptor antagonist; therapeutic potential as anxiolytic, antidepressant anti-migraine, atypical antipsychotic	↑ number of USVs [19]
sodium nitroprusside	inorganic salt; vasodilator	↑ number of USVs [42]
triazolam	benzodiazepine; anxiolytic	↓ number of USVs [18]
zolpidem	imidazopyridine; anxiolytic-hypnotic	↓ number of USVs [17,18]

Table 1

Conditioned foot-shock model of anxiety and fear		
Drug	Pharmacological Class	Effect observed
alpidem	imidazopyridine; anxiolytic	— number of USVs [50]
alprazolam	benzodiazepine; anxiolytic	↓ number of USVs, highly effective [50]
bretazenil	imidazopyrrolobenzodiazepine; anxiolytic,	— number of USVs [50]
bupirone	azapirone, 5HT _{1A} receptor agonist; anxiolytic	↓ number of USVs [50,51]
chlordiazepoxide	benzodiazepine; anxiolytic	— number of USVs; scarcely effective [50] ↓ number of USVs [85]
chlorimipramine	tricyclic; antidepressant	↓ number of USVs [50]
citalopram	SSRI; antidepressant with anxiolytic properties	↓ number of USVs, highly potent [57]
clonidine	central α_2 receptor agonist; antihypertensive with anxiolytic properties	↓ number of USVs [50]
clozapine	dibenzodiazepine; atypical antipsychotic	↓ number of USVs [85]
desipramine	tricyclic; antidepressant	— number of USVs [50]
diazepam	benzodiazepine; anxiolytic	↓ number of USVs [51] — number of USVs; scarcely effective [50]
fluoxetine	SSRI; antidepressant with anxiolytic properties	↓ number of USVs, weak effect [57]
fluvoxamine	SSRI; antidepressant with anxiolytic properties	↓ number of USVs [50,57]
gepirone	azapirone; 5HT _{1A} receptor partial agonist; anxiolytic	↓ number of USVs [52]
haloperidol	D ₂ receptor antagonist; typical antipsychotic	↓ number of USVs, at very high doses [50] — number of USVs; scarcely effective [85]
imipramine	tricyclic; antidepressant	↓ number of USVs [50]
ipsapirone	azapirone; 5HT _{1A} receptor partial agonist; anxiolytic and antidepressant	↓ number of USVs [50]
maprotiline	tetracyclic; antidepressant	— number of USVs [50]
olanzapine	thienobenzodiazepine; atypical antipsychotic	— number of USVs [85]
ondansetron	5HT ₃ receptor partial antagonist; used to treat nausea and vomiting	— number of USVs [50]
paroxetine	SSRI; antidepressant with anxiolytic properties	↓ number of USVs, highly potent [57]
sertraline	SSRI; antidepressant with anxiolytic properties	↓ number of USVs [57]
yohimbine	α_2 receptor antagonist and weak monoamine oxidase inhibitor; used to treat sexual dysfunction	↓ number of USVs [50]
zolpidem	imidazopyridine; anxiolytic-hypnotic	— number of USVs [50]
Stress induced by drug withdrawal after chronic treatment		
diazepam	benzodiazepine; anxiolytic	↑ number of USVs [63,64]
morphine	opiate; analgesic with abuse potential	↑ number of USVs [67]
Experimental models of pain		
amitriptyline	tricyclic; antidepressant	— number of USVs in the tail electric pulse model [75]
aspirin	NSAID; analgesic, anti-inflammatory	— number of USVs, in the tail electric pulse model [75] — number of USVs, in social interactions performed by arthritic rats treated with Freund's adjuvant [80]
ketoprofen	NSAID; analgesic, anti-inflammatory	↓ number of USVs in social interactions performed by arthritic rats treated with Freund's adjuvant [13]
morphine	opiate; analgesic with abuse potential	↓ number of USVs in rats with formalin-induced paw pain [74] ↓ number of USVs in the tail electric pulse model [75]
paracetamol	mild analgesic, antipyretic	↓ number of USVs in the tail electric pulse model [75]
piroxicam	NSAID; analgesic, anti-inflammatory	↓ number of USVs in social interactions performed by arthritic rats treated with Freund's adjuvant [13]
rofecoxib	NSAID; analgesic, anti-inflammatory	↓ number of USVs in social interactions performed by arthritic rats treated with Freund's adjuvant [13]

Table 2

50-kHz USVs Subtype	Features	AMPH (2)	MDMA (5)	MDMA (10)	MDMA (15)	MOR (1)	MOR (2.5)	MOR (5)	NIC (0.1)	NIC (0.2)	NIC (0.4)
Flat	<i>USVs bearing a near-constant frequency</i>	↑*	↓	↓	↓	↓	↓	↓	↓	↓	↓
Trill	<i>USVs displaying a rapid frequency oscillation, usually appearing as a sinusoid oscillation</i>	↑*	↓	-	-	-	-	-	-	-	-
Flat + Trill	<i>USVs composed by a trill and a flat vocalization</i>	↑*	-	-	-	-	-	-	-	-	-
Upward Ramp	<i>USVs displaying a monotonic increase in frequency</i>	↑*	↑	↓	↑	↓	↑	-	↓	↓	↑
Downward Ramp	<i>USVs displaying a monotonic decrease in frequency</i>	↑*	↑	↑*	↑*	↑*	↑	-	↑	↑	↑
Step Up	<i>USVs with an instantaneous frequency change to a higher frequency</i>	↑*	↓	↓	↓	-	-	-	-	-	↓
Step Down	<i>USVs with an instantaneous frequency change to a lower frequency</i>	↑*	-	↑	-	-	-	-	-	-	-
Complex	<i>USVs displaying two or more changes in frequency of at least 3-kHz each</i>	↑*	↓	↑	-	-	↓	↓	↓	-	↓
Composite	<i>USVs combinations other than “Flat + Trill”</i>	↑*	-	-	-	-	-	↑	-	-	-
Split	<i>USVs with a middle component possessing a lower frequency compared with the other components of the USVs</i>	↑*	-	-	-	-	-	-	-	-	-
Multistep	<i>USVs with two or more instantaneous changes in frequency</i>	↑*	-	↓	-	-	-	-	-	-	-
Inverted U	<i>USVs possessing a monotonic increase in frequency followed by a monotonic decrease in frequency, resembling the shape of an inverted U</i>	↑*	↑	↑	↓	↓	-	↓	-	-	-
Short	<i>USVs with duration of less than 12 ms</i>	↑*	↑	↑*	↑	↑	↓	↓	↓	↓	↑

Table 3

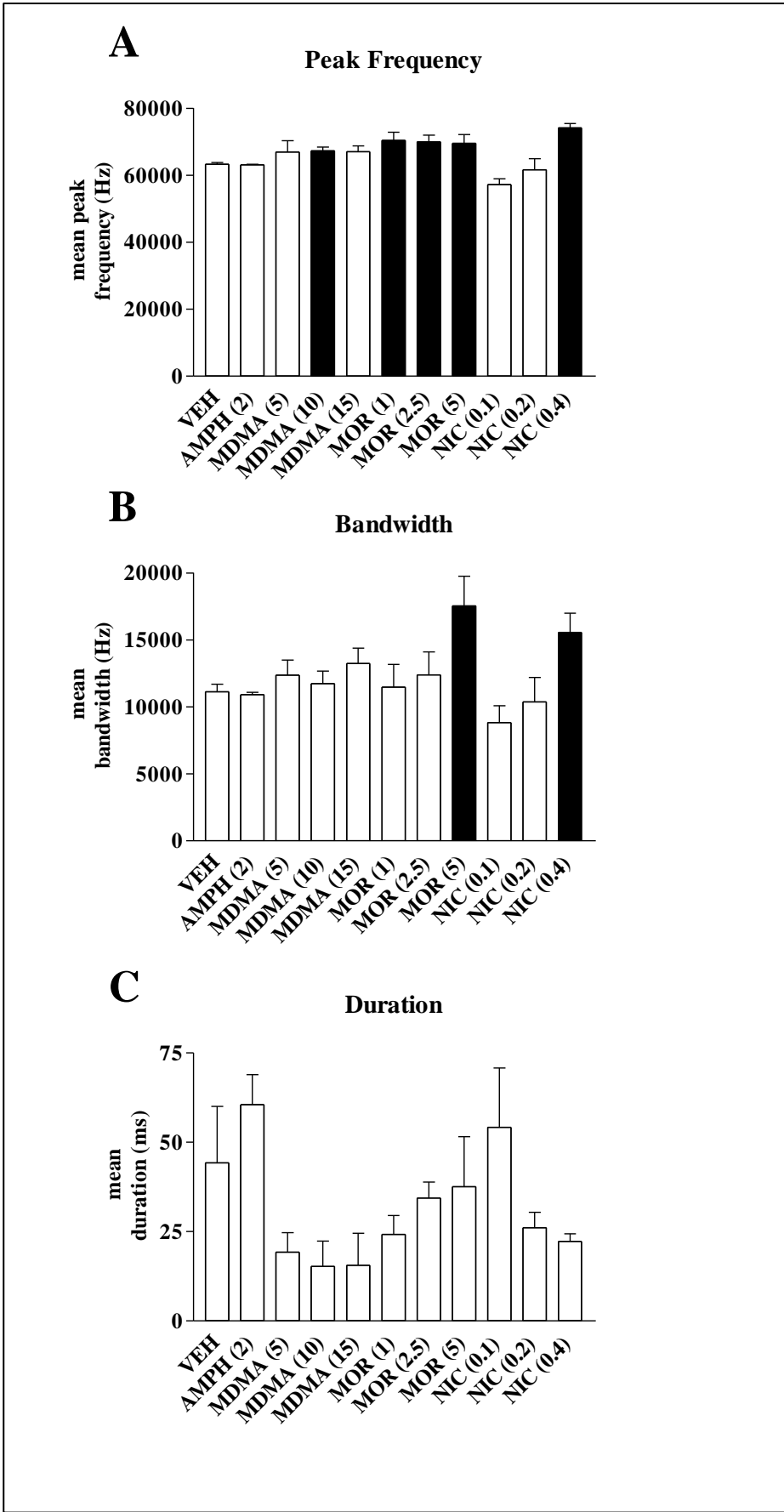


Figure 1

