International Journal of Neuropsychopharmacology (2014), **17**, 429–441. © CINP 2013
doi:10.1017/S1461145713001235

ARTICLE

Direct and long-lasting effects elicited by repeated drug administration on 50-kHz ultrasonic vocalizations are regulated differently: implications for the study of the affective properties of drugs of abuse

Nicola Simola[1], Lucia Frau[1], Antonio Plumitallo[2] and Micaela Morelli[1,3,4]

[1] *Department of Biomedical Sciences, Section of Neuropsychopharmacology, University of Cagliari, Cagliari, Italy*
[2] *Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy*
[3] *Centre of Excellence on Neurobiology of Dependence, University of Cagliari, Cagliari, Italy*
[4] *CNR National Research Council of Italy, Institute of Neuroscience, Cagliari, Italy*

Abstract

Several studies suggest that 50-kHz ultrasonic vocalizations (USVs) may indicate a positive affective state in rats, and these vocalizations are increasingly being used to investigate the properties of psychoactive drugs. Previous studies, however, have focused on dopaminergic psychostimulants and morphine, whereas little is known about how other drugs modulate 50-kHz USVs. To further elucidate the neuropharmacology of 50-kHz USVs, the present study characterized the direct and long-lasting effects of different drugs of abuse, by measuring the number of 50-kHz USVs and their 'trill' subtype emitted by adult male rats. Rats received repeated administrations of amphetamine (2 mg/kg, i.p.), 3,4-methylenedioxymethamphetamine (MDMA, 7.5 mg/kg, i.p.), morphine (7.5 mg/kg, s.c.), or nicotine (0.4 mg/kg, s.c.), on either consecutive or alternate days (five administrations in total) in a novel environment. Seven days later, rats were re-exposed to the drug-paired environment, subjected to USVs recording, and then challenged with the same drug. Finally, 7 d after the challenge, rats were repeatedly exposed to the drug-paired environment and vocalizations were measured. Amphetamine was the only drug to stimulate 50-kHz USVs and 'trill' subtype emission during administration and challenge. Conversely, all rats emitted 50-kHz USVs when re-exposed to the test cage, and this effect was most marked in morphine-treated rats, and less evident in nicotine-treated rats. This study demonstrates that the direct and long-lasting effects of drugs on 50-kHz USVs are regulated differently, providing a better understanding of the usefulness of these vocalizations in the study of psychoactive drugs.

Received 12 July 2013; Reviewed 28 July 2013; Revised 21 September 2013; Accepted 24 September 2013; First published online 21 October 2013

Key words: Amphetamine, MDMA, morphine, nicotine, reward.

Introduction

Rats emit ultrasonic vocalizations (USVs) in response to a wide range of stimuli, and a link between USVs and emotional state has been suggested (Knutson et al., 2002; Covington and Miczek, 2003; Panksepp, 2005; Wöhr et al., 2005; Brudzynski, 2007, 2013; Schwarting et al., 2007; Willey and Spear, 2013). In this regard, it has been proposed that the so-called '50-kHz' USVs, with a frequency range of 35–90 kHz (Portfors, 2007), indicate a positive effect, as they are preferentially emitted in response to, or in anticipation of, pleasurable stimuli, such as mating and non-aggressive social encounters (Knutson et al., 1999; Brudzynski, 2005; Burgdorf et al., 2008, 2011). Interestingly, 50-kHz USVs can also be observed following the administration of rewarding drugs (Wintink and Brudzynski, 2001; Mu et al., 2009; Simola et al., 2012). Accordingly, independent investigations have suggested that 50-kHz USVs emission may be a straightforward tool to study the effects of drugs on affect and motivation (Ahrens et al., 2009; Mu et al., 2009; Williams and Undieh, 2010; Hamed et al., 2012).

Although this hypothesis is intriguing, previous studies by us and others have demonstrated that drugs of abuse may have different effects on 50-kHz USVs. Investigations using acute drug administration found that the dopaminergic psychostimulants amphetamine, cocaine, and methylphenidate significantly stimulated 50-kHz USVs, whereas caffeine, 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy'), morphine, and nicotine did not

Address for correspondence: Dr N. Simola, Department of Biomedical Sciences, Section of Neuropsychopharmacology, University of Cagliari, Via Ospedale, 72, 09124, Cagliari, Italy.
Tel.: +39 -070-6758687 *Fax*: +39-070-6758665
Email: nicola.simola@gmail.com

(Wintink and Brudzynski, 2001; Simola et al., 2010, 2012; Williams and Undieh, 2010; Sadananda et al., 2012). Conflicting results have also been obtained by studies using repeated drug administration. Thus, a progressive increase in 50-kHz USVs emission has been observed in rats treated with amphetamine or cocaine (Ahrens et al., 2009; Mu et al., 2009). Conversely, others found mixed effects of amphetamine and no effect of morphine (Taracha et al., 2012; Wright et al., 2012). Moreover, little is known about the effects of repeated administration of drugs other than dopaminergic psychostimulants and morphine on 50-kHz USVs modulation, and further characterization of this is required. Importantly, investigations should be done under comparable conditions, to avoid potential confounding factors related to differences in the experimental procedures used.

This study evaluated USVs emissions in rats subjected to repeated treatment (five administrations) with amphetamine, MDMA, morphine, or nicotine. USVs elicited on drug treatment and challenge, administered 7 d after drug discontinuation, were recorded, together with USVs, emitted on re-exposure to the drug-paired environment. To ascertain the effects of drug administration protocol on USVs, rats received drugs on either consecutive or alternate days. Moreover, as different subtypes of 50-kHz USVs exist and 'trill' vocalizations are envisioned as those most closely linked to pleasurable stimuli (Burgdorf et al., 2008; Ahrens et al., 2009; Wright et al., 2010), modifications involving 'trill' USVs, either stimulated by drugs or emitted on test cage re-exposure were evaluated. This approach was used to characterize the effects of different drugs of abuse on 50-kHz USVs, and to gain further insight into the relevance of these vocalizations in the study of psychoactive drugs.

Method

Subjects

Male Sprague–Dawley rats (Harlan, Italy) weighing 275–300 g were used. Rats were housed in groups of four or five in standard polycarbonate cages with sawdust bedding, and maintained on a 12-h light/dark cycle (lights on at 08:00 h). Rats had free access to food (standard laboratory chow) and tap water, except during the experiments, which took place from 10:00 to 15:00 h.

All experiments were conducted in accordance with the guidelines for care and use of experimental animals of the EU directives (2010/63/EU; L.276; 22/09/2010), and with the guidelines approved by the Ethical Committee of the University of Cagliari. Efforts were made to minimize the number of animals used and maximize humane treatment.

Drugs

D-Amphetamine (sulfate) and nicotine (bitartrate) were purchased from Sigma–Aldrich (Italy). MDMA was synthesized at the Department of Life and Environmental Sciences of the University of Cagliari, as described elsewhere (Frau et al., 2013). Morphine (hydrochloride) was purchased from Franchini Prodotti Chimici srl (Italy). All drugs were dissolved in distilled water. Morphine (7.5 mg/kg) and nicotine (0.4 mg/kg) were injected subcutaneously (s.c.), in a volume of 1 ml/kg. Nicotine solutions were neutralized with NaOH (1 N) before being administered (final pH=7). Amphetamine (2 mg/kg) and MDMA (7.5 mg/kg) were administered intraperitoneally (i.p.), in a volume of 3 ml/kg. The doses of the drugs used in this study were selected based on our previous study evaluating the acute effects of the same drugs (Simola et al., 2012), and on preliminary experiments showing that repeated administration of MDMA, morphine, or nicotine at doses lower than those used here failed to significantly stimulate 50-kHz USVs in rats.

Experimental procedure

The experiments were designed based on our previous studies (Simola et al., 2010, 2012). Rats were handled daily (5 min) for 2 d before experimentation. Experiments consisted of seven phases: (1) habituation to the test cage; (2) acute administration of vehicle in the test cage, to quantify basal USVs; (3) repeated drug or vehicle administration in the test cage on either consecutive or alternate days (five administrations in total), and USVs recording on first and fifth administration; (4) drug withdrawal in the home cage; (5) evaluation of USVs elicited by test cage re-exposure, followed by drug challenge and recording of drug-stimulated USVs; (6) drug withdrawal in the home cage; (7) repeated test cage re-exposure to evaluate the persistence and extinction of USVs. Locomotor activity was measured throughout the experiments by means of automated counters (Opto-Varimex; Columbus Instruments, Columbus, OH, USA). Figure 1 shows the experimental plan. An intermittent administration regimen was employed to attenuate the emergence of tolerance (Simola et al., 2006), and evaluate whether the administration protocol may influence the effects of drugs on USVs. Separate groups of rats were treated with amphetamine (2 mg/kg, i.p.), MDMA (7.5 mg/kg, i.p.), morphine (7.5 mg/kg, s.c.), or nicotine (0.4 mg/kg, s.c.). Rats were randomly assigned to an experimental group, and vehicle-treated rats served as controls. Groups were composed as follows: amphetamine, n=6/6; MDMA, n=6/7; morphine, n=8/8; nicotine, n=6/6; vehicle, n=6/6, where n represents the number of rats receiving the drug in consecutive/alternate administration.

USVs recording

USVs recording took place in a quiet room. Each rat was individually placed in a Plexiglas cylinder (diameter, 25 cm; height, 30 cm) enclosed by four cardboard walls (height, 65 cm; distance from the cylinder, 15 cm). The bottom of the cylinder was covered with sawdust taken

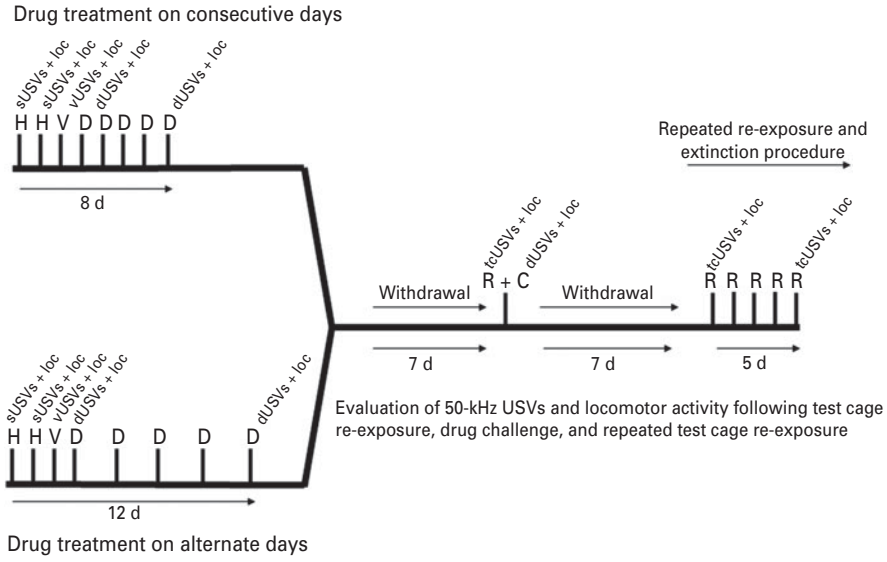

Fig. 1. Experimental plan. Rats were habituated to the test cage for 2 d (15 min, twice a day), then received an acute vehicle administration to evaluate basal 50-kHz ultrasonic vocalizations (USVs). Starting from the day after vehicle administration, rats underwent repeated drug treatment, on either consecutive or alternate days. During habituation to the test cage, spontaneous USVs (sUSVs) were evaluated. Afterwards, evaluation of 50-kHz USVs was performed after vehicle (vUSVs) or drug administration (dUSVs), or re-exposure to the test cage (tcUSVs) at the time points indicated in the figure. Locomotor activity was evaluated throughout habituation and experiments. C=drug challenge; D=drug administration in the test cage; H=habituation to the test cage; R=re-exposure to the test cage; V=vehicle administration.

from the individual rat home cage, to attenuate the influence of a novel environment on USVs emission (Natusch and Schwarting, 2010). The cylinder was covered with a lid equipped with an ultrasonic microphone bearing high directional properties (CM16/CMPA, Avisoft, Germany), and connected to an ultrasound-recording device (UltraSoundGate 116Hb, Avisoft, Germany). During recordings, intensity gain was kept at the same level for all the rats. USVs stimulated by either drug or vehicle were recorded for 1 h, whereas USVs emitted on re-exposure to the drug-paired environment were recorded for 10 min (Fig. 1).

Vocalizations emitted by morphine-treated rats on test cage re-exposure

Rats receiving morphine on alternate days exhibited a pronounced and long-lasting emission of 50-kHz USVs on test cage re-exposure. Therefore, an additional group of morphine-treated rats was employed to ascertain the conditioned nature of these vocalizations. Rats (n=6) received four morphine administrations (7.5 mg/kg. s.c.) in the home cage, alternating with four vehicle administrations in the test cage. The day after the last vehicle administration, rats received a fifth morphine administration in the test cage, and drug-stimulated USVs were recorded. USVs emitted on test cage re-exposure were measured 7 d later; afterwards these rats underwent drug challenge and USVs extinction.

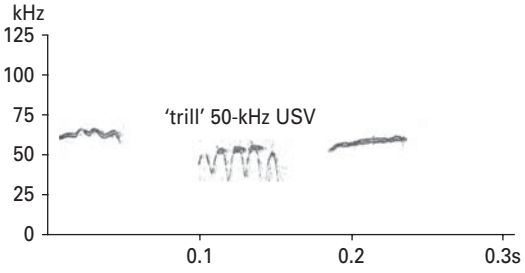

Fig. 2. Example of sonograms of 50-kHz ultrasonic vocalizations (USVs).

Data collection, analysis, and statistics

The software SASLab Pro 4.52 (Avisoft, Germany) was used to convert USVs recordings into spectrograms with these settings: 512 FFT-length, Hamming window, and 75% overlap frame set-up (Wöhr and Schwarting, 2009; Simola et al., 2012). Spectrograms were visually inspected by an experienced experimenter, then processed as described elsewhere (Simola et al., 2012). After manual cleaning of all signals that could not be univocally classified as vocalizations, the number of 50-kHz USVs was calculated by SASLab Pro 4.52. For classification of 'trill' 50-kHz USVs, each spectrogram was visually inspected three times, separated by a 7-d interval. USVs that were not uniformly classified over the three evaluations were discarded. USVs were classified as 'trill' vocalizations according to Wright et al. (2010) (Fig. 2). The present study observed similar emissions of

Table 1. Locomotor activity measured during drug treatment and re-exposure to the test cage. Locomotor activity counts measured in rats that received repeated treatment with amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, or nicotine on consecutive days (upper panel) and on alternate days (lower panel). Locomotor activity counts were measured during the first and fifth drug administration, on re-exposure to the test cage immediately before drug challenge, and after drug challenge

Locomotor activity counts after drug treatment on consecutive days and challenge

Treatment	First administration	Fifth administration	Drug challenge	Re-exp. to the drug-paired environment
Amphetamine	4678.5±514.6*	7354.5±719.6*	6681±751.1*	711±61.5*
MDMA	7366.7±1030.6*	5543.8±447.1*	6389.5±728.9*	538.7±65.8
Morphine	483±135.8*	645.8±136	525.5±92.1	518.1±39.6
Nicotine	1167.7±174*	1956.3±156.9*	1705±141.2*	457.7±76.7
Vehicle	851±139.8	666.2±104.2	613.8±54.2	464.7±69.3

Locomotor activity counts after drug treatment on alternate days and challenge

Treatment	First administration	Fifth administration	Challenge	Re-exp. to the drug-paired environment
Amphetamine	4839.2±587.3*	7770.7±901.9*	6253.2±668.4*	717±92.7*
MDMA	4789±218.4*	4040±447.3*	5187.4±472.2*	575.4±34.4*
Morphine	177.4±36*	676±72.2	602±62.2	581±29.5*
Nicotine	1080.2±103.4*	1683.3±194.7*	1357±187.8*	568.8±35.5*
Vehicle	669±172.8	887±130.4	592.8±55.1	422.7±43.2

MDMA=3,4-methylenedioxymethamphetamine; re-exp.=re-exposure.
* $p<0.05$ compared with vehicle-treated rats.

drug-stimulated 50-kHz USVs in rats administered drugs on consecutive and alternate days. Conversely, only the latter rats displayed a significant emission of 50-kHz USVs when re-exposed to the test cage. Therefore, to compare the direct and long-lasting effects of drugs, analysis of 'trill' 50-kHz USVs was narrowed to rats treated on alternate days. USVs data obtained in the present study were found to be normally distributed, therefore parametric statistics were employed. Means±S.E.M. of the number of total and 'trill' 50-kHz USVs, of the percentage of 'trill' USVs (over total USVs), and of locomotor activity counts were calculated. Time-course evaluation of drug-stimulated 50-kHz USVs was also performed. Data were analyzed by means of two-tailed Student's *t* test, two-way (treatment × day), or three-way (treatment × day × time), analysis of variance (ANOVA), followed by Tukey's *post-hoc* test when appropriate. Significance was set at $p<0.05$ for each analysis. Statistical analysis was performed with Statistica for Windows (StatSoft, USA).

Results

Effect of drugs and re-exposure to the test cage on locomotor activity

The doses of amphetamine, MDMA, and nicotine used in this study significantly stimulated locomotor activity, compared with vehicle administration. The dose of morphine used in this study did not elicit motor suppression (except on the first administration) and sedation. When re-exposed to the test cage at 7 d from drug discontinuation, rats treated on alternate days displayed a significantly higher locomotor activity than vehicle-treated rats, and this was evident for all the drugs evaluated (Table 1). This was no longer present 7 d after drug challenge (data not shown).

Emission of 50-kHz USVs stimulated by acute vehicle, and emission of 22-kHz USVs

Acute vehicle administration 1 d before drug treatments scarcely stimulated 50-kHz USVs and locomotion, with no differences among the experimental groups (data not shown). Moreover, 22-kHz USVs, which are thought to reflect negative affective states (Brudzynski, 2007), were emitted by two rats administered nicotine, and one administered amphetamine, which were excluded from the analysis.

Emission of 50-kHz USVs stimulated by repeated drug administration

Amphetamine

Rats repeatedly treated with amphetamine (2 mg/kg, i.p.), on either consecutive or alternate days, emitted significantly more 50-kHz USVs than vehicle-treated rats (Fig. 3*a*). Two-way ANOVA for consecutive-day amphetamine revealed an effect of treatment ($F_{1,10}=12.42$, $p=0.03$), but no effect of day ($F_{1,10}=0.31$, $p=0.26$), and no treatment × day interaction ($F_{1,10}=1.23$, $p=0.25$).

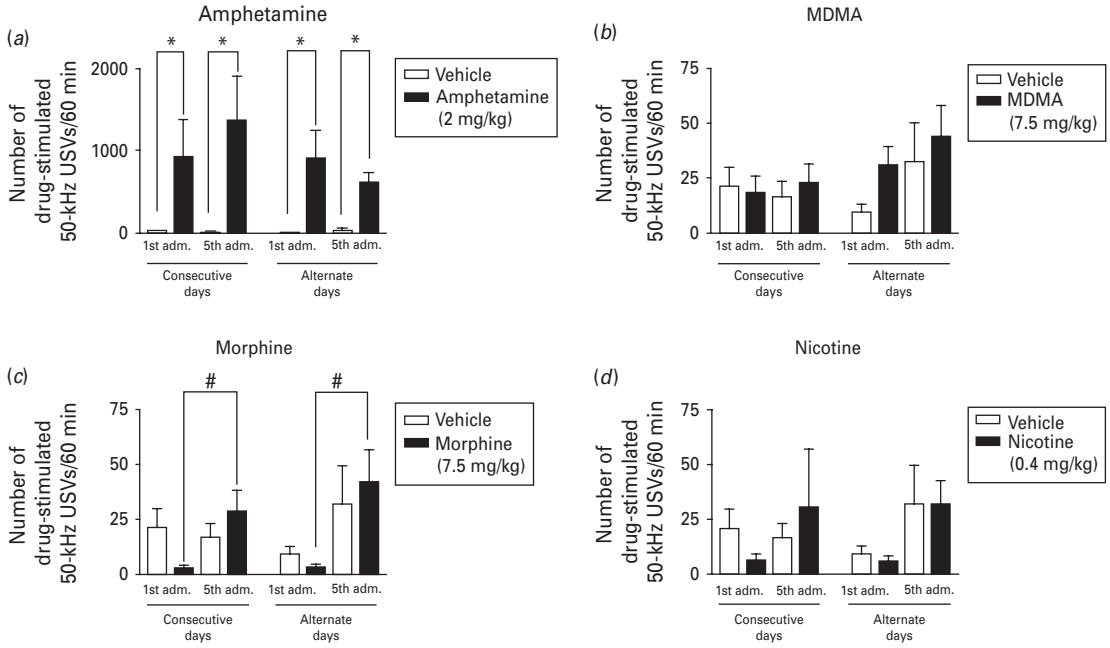

Fig. 3. Effect of repeated treatment with amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, or nicotine on the emission of 50-kHz ultrasonic vocalizations (USVs). Rats received five drug administrations on either consecutive or alternate days. Each rat was administered only one given drug. USVs were recorded during the first and fifth drug administrations. *$p<0.05$ compared with vehicle-treated rats. #$p<0.05$ compared with morphine-treated rats. adm.=administration.

Similarly, two-way ANOVA for alternate-day amphetamine showed an effect of treatment ($F_{1,10}=11.37$ $p=0.01$), but no effect of day ($F_{1,10}=1.18$, $p=0.30$), and no treatment × day interaction ($F_{1,10}=1.59$, $p=0.23$). No significant differences in 50-kHz USVs emissions were observed between rats administered amphetamine on consecutive or alternate days (Fig. 3*a*, Supplementary Table S1).

MDMA

Repeated MDMA (7.5 mg/kg, i.p.) administration, on either consecutive or alternate days, stimulated 50-kHz USVs emissions similar to that recorded in vehicle-treated rats. The 50-kHz USVs emissions of MDMA-treated rats were similar for consecutive- and alternate-day administration (Fig. 3*b*, Supplementary Table S1).

Morphine

Rats repeatedly treated with morphine (7.5 mg/kg, s.c.) showed an emission of 50-kHz USVs not significantly different from that of vehicle-treated rats. Nevertheless, significant modifications in morphine-stimulated vocalizations occurred during drug administration (Fig. 3*c*). Two-way ANOVA for consecutive-day morphine revealed no effect of treatment ($F_{1,12}=0.13$, $p=0.72$), but showed an effect of day ($F_{1,12}=4.63$, $p=0.04$), and a treatment × day interaction ($F_{1,12}=7.06$, $p=0.02$). As for alternate-day morphine, two-way ANOVA indicated no effect of treatment ($F_{1,12}=0.02$, $p=0.90$), and no treatment × day interaction ($F_{1,12}=0.53$, $p=0.47$), but revealed an effect of day ($F_{1,12}=7.87$, $p=0.02$). Tukey's *post-hoc* test showed that rats treated with morphine on either consecutive or alternate days emitted significantly more 50-kHz USVs on the fifth drug administration. When 50-kHz USVs emissions of rats treated with morphine on consecutive days and alternate days were compared, two-way ANOVA showed an effect of day ($F_{1,14}=15.5$, $p=0.01$), but no effect of treatment ($F_{1,14}=0.51$, $p=0.49$), and no treatment × day interaction ($F_{1,14}=0.57$, $p=0.46$) (Fig. 3*c*).

Nicotine

Repeated treatment with nicotine (0.4 mg/kg, s.c.), on either consecutive or alternate days, stimulated 50-kHz USVs emissions similar to that observed in vehicle-treated rats. This effect was comparable between rats receiving nicotine on consecutive and alternate days (Fig. 3*d*, Supplementary Table S1).

Emission of 50-kHz USVs following re-exposure to the drug-paired environment or drug challenge

Amphetamine

On test cage re-exposure 7 d after treatment discontinuation, rats repeatedly treated with amphetamine on consecutive days displayed an emission of 50-kHz USVs not significantly different from that of vehicle-treated rats, although a trend towards an increase in this effect was present. Conversely, rats repeatedly treated with

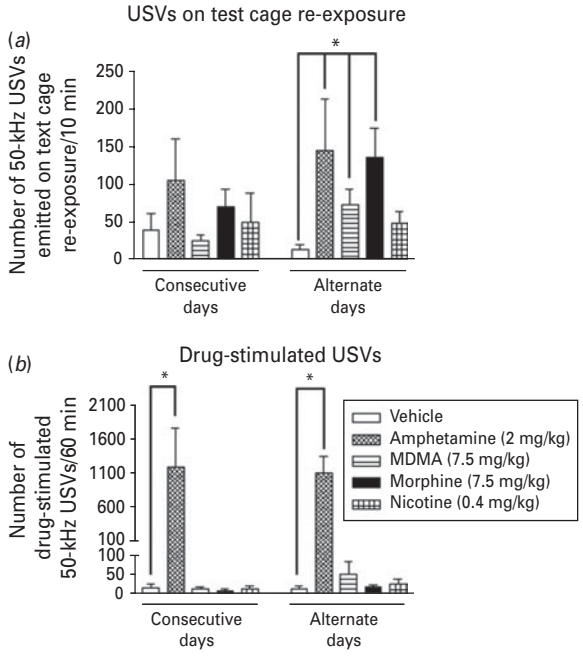

Fig. 4. Emission of 50-kHz ultrasonic vocalizations (USVs) after re-exposure to the test cage and drug challenge by rats previously treated with amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, or nicotine. Rats treated on either consecutive or alternate days were first re-exposed to the test cage, 7 d after treatment discontinuation, and vocalizations were recorded (*a*). Thereafter, rats received a challenge with their respective drug, and drug-stimulated 50-kHz USVs were recorded (*b*). *$p<0.05$ compared with vehicle-treated rats.

amphetamine on alternate days emitted significantly more 50-kHz USVs compared with vehicle-treated rats ($t=2.31$, df=10, $p=0.04$) (Fig. 4*a*).

Amphetamine challenge (2 mg/kg, i.p.), administered after test cage re-exposure, elicited a significantly higher 50-kHz USVs emission compared with vehicle challenge. Student's *t* test revealed this effect for both rats treated with amphetamine on consecutive ($t=4.78$, df=10, $p=0.01$), and alternate days ($t=4.49$, df=10, $p=0.01$) (Fig. 4*b*). However, amphetamine challenge failed to further elevate 50-kHz USVs emission compared with previous amphetamine treatments (Supplementary Table S2).

MDMA

Rats repeatedly administered MDMA on consecutive days showed a 50-kHz USVs emission comparable with vehicle-treated rats, on test cage re-exposure 7 d after treatment discontinuation. Conversely, rats repeatedly administered MDMA on alternate days emitted more 50-kHz USVs than vehicle-treated rats when re-exposed to the test cage ($t=2.15$, df=11, $p=0.04$) (Fig. 4*a*).

Rats challenged with MDMA (7.5 mg/kg, i.p.) after test cage re-exposure emitted 50-kHz USVs similar to vehicle-challenged rats, although a trend towards an increase in this effect was present in rats treated with MDMA on alternate days (Fig. 4*b*). MDMA challenge did not further elevate 50-kHz USVs emission compared with previous MDMA treatments (Supplementary Table S2).

Morphine

On test cage re-exposure 7 d after treatment discontinuation, rats repeatedly treated with morphine on consecutive days showed a 50-kHz USVs emission not significantly different from that of vehicle-treated rats, although a trend towards an increase in this effect was observed. Conversely, rats repeatedly treated with morphine on alternate days emitted significantly more 50-kHz USVs than vehicle-treated rats on test cage re-exposure ($t=2.69$, df=12, $p=0.02$) (Fig. 4*a*).

Challenge with morphine (7.5 mg/kg, s.c.) after test cage re-exposure, stimulated an emission of 50-kHz USVs similar to that elicited by vehicle challenge, irrespective of the previous morphine administration protocol (Fig. 4*b*). Morphine challenge failed to further elevate 50-kHz USVs compared with previous morphine treatments (Supplementary Table S2).

Nicotine

Rats repeatedly administered nicotine on consecutive days showed a 50-kHz USVs emission comparable with that of vehicle-treated rats, on test cage re-exposure 7 d after treatment discontinuation. Similar results were observed in rats receiving repeated nicotine on alternate days, although the difference with vehicle-treated rats was close to significance ($p=0.07$) (Fig. 4*a*).

Challenge with nicotine (0.4 mg/kg, s.c.), administered after test cage re-exposure, elicited a 50-kHz USVs emission comparable with that stimulated by vehicle challenge, irrespective of the previous nicotine administration protocol (Fig. 4*b*). Nicotine challenge did not further elevate 50-kHz USVs compared with previous nicotine treatments (Supplementary Table S2).

Time-course of drug-stimulated 50-kHz USVs during repeated administration and challenge

Time-course analysis of drug-stimulated 50-kHz USVs during repeated treatment revealed significant time-point differences between amphetamine- and vehicle-treated rats. No differences with vehicle-treated rats were found for the other drugs evaluated. Three-way ANOVA for consecutive-day amphetamine revealed significant effects of treatment ($F_{1,20}=10.16$, $p=0.01$), time ($F_{5,100}=2.40$, $p=0.04$), and treatment × time interaction ($F_{1,20}=2.42$, $p=0.02$). Effects of day, treatment × day interaction, and treatment × day × time interaction were not significant. Three-way ANOVA for alternate-day amphetamine showed significant effects of treatment ($F_{1,20}=16.77$,

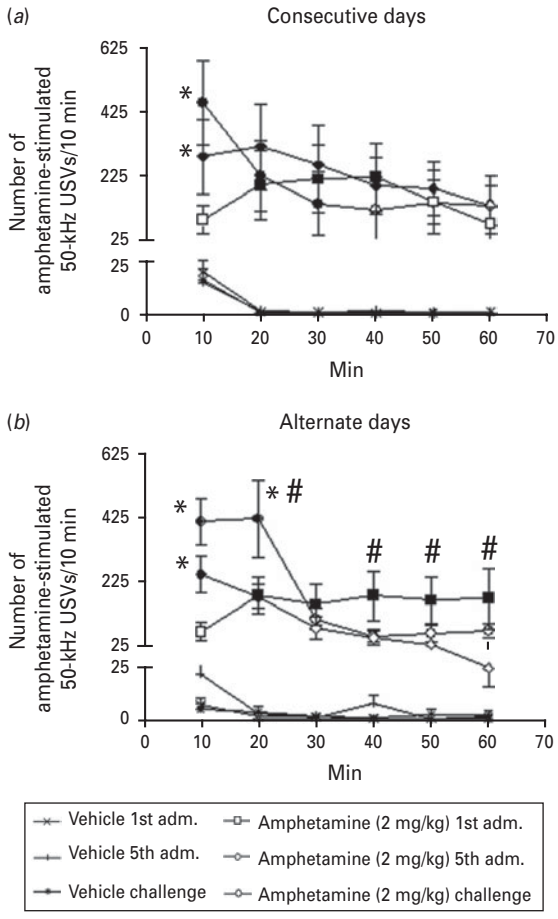

Fig. 5. Time-course of drug-stimulated 50-kHz ultrasonic vocalizations (USVs) in rats administered amphetamine on consecutive or alternate days. Vocalizations stimulated by amphetamine or vehicle are reported for the 1 h recording time, subdivided in 6 intervals of 10 min each. Data are presented for the first and fifth administration, and challenge. Filled symbols indicate a significant difference with the corresponding time-point of vehicle-treated rats. $^{*}p<0.05$ compared with the first amphetamine administration. $^{\#}p<0.05$ compared with the fifth amphetamine administration. adm. =administration.

$p=0.01$), time ($F_{5,100}=2.31$, $p=0.04$), day × time interaction ($F_{1,20}=6.50$, $p=0.01$), and treatment × day × time interaction ($F_{15,100}=5.64$, $p=0.01$). Effects of day, and treatment × day interaction were not significant. Time-point differences revealed by Tukey's *post-hoc* test are reported in Fig. 5.

Similar results were obtained for the time-course of 50-kHz USVs stimulated by drug challenge, with amphetamine being the only drug that displayed significant time-point differences compared with vehicle, as shown by two-way ANOVA. Moreover, two-way ANOVA comparisons followed by Tukey's *post-hoc* test revealed significant time-point differences between amphetamine challenge and previous amphetamine administrations, with a shift in the maximal number of 50-kHz USVs emitted to earlier time points over the course of treatment (Fig. 5). This effect was observed for both continuous and alternate amphetamine administration.

Emission of 50-kHz USVs on repeated re-exposure to the drug-paired environment

Amphetamine

Rats administered amphetamine, on either consecutive or alternate days, displayed 50-kHz USVs emissions comparable with vehicle-treated rats on repeated test cage re-exposure, starting 7 d after amphetamine challenge. No differences in this effect were observed between rats treated with amphetamine on consecutive or alternate days (Fig. 6*a*, Supplementary Table S1).

MDMA

Rats administered MDMA, on either consecutive or alternate days, showed 50-kHz USVs emissions similar to vehicle-treated rats on repeated test cage re-exposure, starting 7 d after MDMA challenge. This effect was similar between rats on consecutive- and alternate-day MDMA treatment (Fig. 6*b*, Supplementary Table S1).

Morphine

Rats administered morphine on consecutive days displayed an emission of 50-kHz USVs not significantly different from vehicle-treated rats on repeated test cage re-exposure, starting 7 d after morphine challenge. Nevertheless, morphine-treated rats displayed a trend towards higher vocalization on the first re-exposure (Fig. 6*c*).

Conversely, rats treated with morphine on alternate days showed a 50-kHz USVs emission different from that of vehicle-treated rats. Two-way ANOVA revealed an effect of treatment ($F_{1,12}=4.77$, $p=0.04$) and an effect of day ($F_{1,12}=6.00$, $p=0.04$), but no treatment × day interaction ($F_{1,12}=1.6$, $p=0.23$). Tukey's *post-hoc* test indicated that morphine-treated rats exhibited significantly more vocalizations on the first re-exposure to the test cage (Fig. 6*c*).

Moreover, two-way ANOVA indicated an effect of day ($F_{1,14}=9.18$, $p=0.01$), but no effect of treatment ($F_{1,14}=0.48$, $p=0.49$), and no treatment × day interaction ($F_{1,14}=3.18$, $p=0.09$) when the 50-kHz USVs emissions on repeated test cage re-exposure were compared between rats administered morphine on consecutive and alternate days (Fig. 6*c*).

Nicotine

Rats administered nicotine, on either consecutive or alternate days, showed 50-kHz USVs emissions not significantly different from that of vehicle-treated rats on repeated test cage re-exposure, starting 7 d after nicotine challenge. Moreover, rats treated with nicotine on consecutive days displayed a 50-kHz USVs emission similar

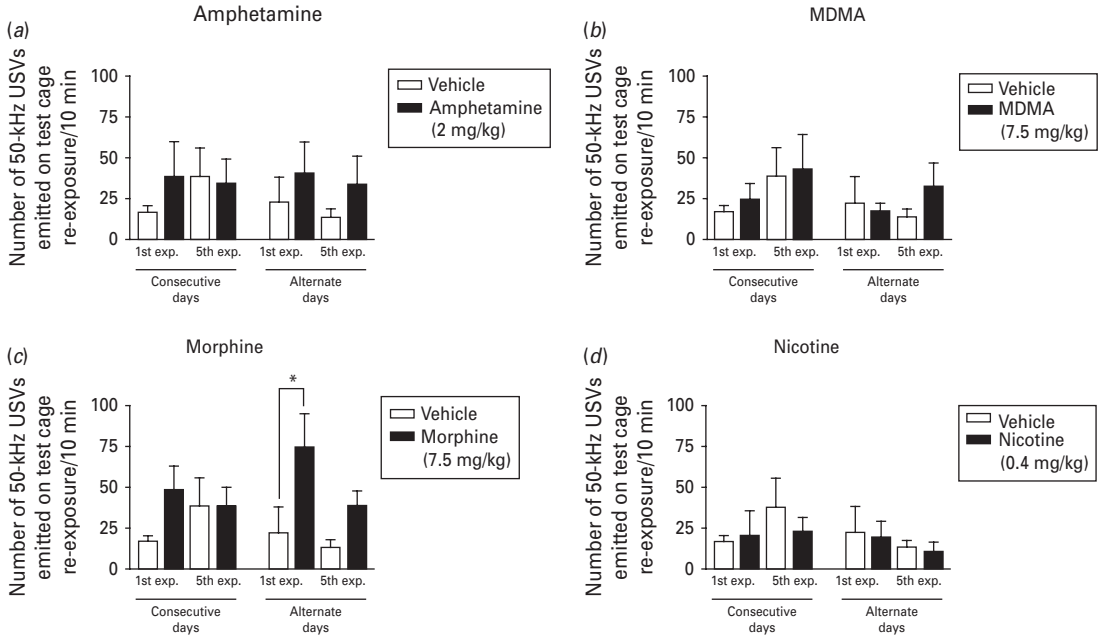

Fig. 6. Emission of 50-kHz ultrasonic vocalizations (USVs) during repeated test cage re-exposure. Starting at 7 d after drug challenge, rats previously treated with amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, or nicotine, on either consecutive or alternate days, were re-exposed to the test cage (10 min) once a day for 5 consecutive days, and vocalizations were recorded on the first and fifth exposures. *$p<0.05$ compared with vehicle-treated rats. exp.=exposure.

to that of rats on alternate-day administration (Fig. 6*d*, Supplementary Table S1).

Emission of 50-kHz USVs following drug administration and test cage re-exposure in rats treated with morphine in the home cage

In this study, rats administered morphine on alternate days displayed the most pronounced and long-lasting 50-kHz USVs emission on test cage re-exposure. Therefore, to ascertain the conditioned nature of these vocalizations, a group of rats that received morphine in the home cage was later exposed to the test cage, and the emission of 50-kHz USVs evaluated. Rats received repeated morphine (7.5 mg/kg, s.c., four administrations) in the home cage (MHC rats), alternated with vehicle in the test cage every other day. When given a fifth morphine administration in the test cage, MHC rats displayed a modest drug-stimulated 50-kHz USVs emission, similar to that observed in rats repeatedly treated with either vehicle ($t=0.51$, df=10, $p=0.62$) or morphine in the test cage (MTC rats) on alternate days ($t=0.96$, df=12, $p=0.36$) (Fig. 7*a*). MHC rats emitted a low number of 50-kHz USVs on test cage re-exposure 7 d after drug discontinuation, immediately before morphine challenge. This effect was comparable with that observed in vehicle-treated rats ($t=0.39$, df=10, $p=0.71$), but significantly lower than that observed in MTC rats ($t=2.32$, df=12, $p=0.04$) (Fig. 7*b*). Following a challenge with morphine (7.5 mg/kg, s.c.) in the test cage, MHC rats displayed a scarce vocalization, which was similar to that recorded from either

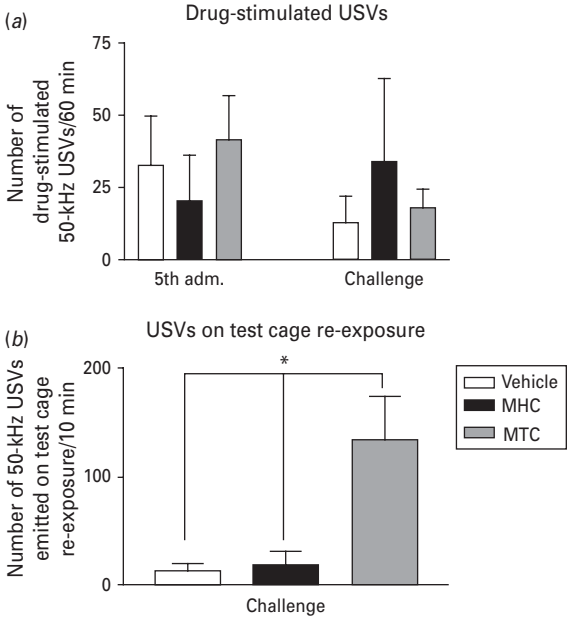

Fig. 7. Emission of 50-kHz ultrasonic vocalizations (USVs) after drug administration and re-exposure to the test cage by rats previously treated with morphine in the home cage. Drug-stimulated 50-kHz USVs were recorded after the fifth morphine administration in the test cage, and after drug challenge given 7 d later (*a*). Rats were re-exposed to the test cage immediately before morphine challenge, and 50-kHz USVs were recorded (*b*). *$p<0.05$. adm.=administration. MHC=morphine in home cage; MTC=morphine in test cage.

Table 2. Emission of 'trill' 50-kHz ultrasonic vocalizations (USVs). The upper panel reports the drug-stimulated 'trill' USVs measured during repeated treatment with amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), morphine, or nicotine, and drug challenge. The lower panel reports the 'trill' USVs measured before the administration of drug challenge, and during repeated test cage re-exposure

Emission of 'trill' 50-kHz USVs on repeated drug treatment and challenge

	First administration		Fifth administration		Challenge	
Treatment	Number	Perc.	Number	Perc.	Number	Perc.
Amphetamine	109.7±41.9*	9.6±3.2	27±13.6*	5.2±3.0	49.8±16.4*	5.6±2.5
MDMA	1.7±0.9	6.9±3.6	0.1±0.1	0.5±0.5	0±0	0±0
Morphine	0.1±0.1	2.1±2.1	2.3±1.3	3.1±1.2	0.1±0.1	0.2±0.2
Nicotine	0±0	0±0	0±0	0±0	0±0	0±0
Vehicle	0.3±0.2	3±2.1	0.5±0.5	0.5±0.5	0.2±0.2	0.3±0.3

Emission of 'trill' 50-kHz USVs on re-exposure to the drug-paired environment

	Challenge day		First repeated re-exp.		Fifth repeated re-exp.	
Treatment	Number	Perc.	Number	Perc.	Number	Perc.
Amphetamine	3±1.2	3.9±2.5	0.3±0.3	0.4±0.4	1.7±1.7	1.4±1.4
MDMA	2.9±2.5	1.9±1.4	0.2±0.2	1.5±1.5	1.7±0.8	4.1±2.2
Morphine	8.5±4.4	3.7±1.5	5.1±2.2	5.5±1.7	3±1.2	6.5±2.4
Nicotine	0±0	0±0	0.2±0.2	2.1±2.1	0±0	0±0
Vehicle	0.7±0.7	1.6±1.6	0.3±0.2	1.3±1.1	0.2±0.2	1.3±1.3

MDMA=3,4-methylenedioxymethamphetamine. perc.=percentage; re-exp=re-exposure.
* $p<0.05$ compared with vehicle-treated rats.

vehicle-treated ($t=1.14$, df=10, $p=0.29$) or MTC rats ($t=1.12$, df=12, $p=0.28$) (Fig. 7*a*). Finally, MHC rats showed a vocalization similar to that of vehicle-treated rats on repeated test cage re-exposure (data not shown).

Emission of 'trill' 50-kHz USVs following drug administration on alternate days and re-exposure to the drug-paired environment

Table 2 summarizes the changes in the numbers and percentages of 'trill' 50-kHz USVs recorded during repeated treatment on alternate days with amphetamine, MDMA, morphine, or nicotine, re-exposure to the test cage and drug challenge, and repeated test cage re-exposure. Repeated treatment and challenge with amphetamine (2 mg/kg, i.p.) were the only conditions that elevated the number of 'trill' 50-kHz USVs, compared with repeated vehicle administration. Two-way ANOVA indicated a significant effect of treatment ($F_{1,10}=9.44$, $p=0.02$), but no effect of day ($F_{1,10}=3.51$, $p=0.06$), and no treatment × day interaction ($F_{1,10}=3.52$, $p=0.06$). Moreover, *t* test revealed a significant effect of amphetamine challenge 7 d after drug discontinuation ($t=3.03$, df=10, $p=0.01$). All the other changes observed in the numbers and percentages of 'trill' 50-kHz USVs did not reach statistical significance (Supplementary Tables S3 and S4). The time-course of 'trill' 50-kHz USVs was similar to that of the total 50-kHz USVs, but no significant modifications were observed (data not shown).

Discussion

The present study evaluates the direct and long-lasting effects of repeated administration of amphetamine, MDMA, morphine, or nicotine on 50-kHz USVs. The results obtained demonstrate that amphetamine was the only drug to significantly stimulate 50-kHz USVs. On the other hand, all but nicotine-treated rats displayed a higher 50-kHz USVs emission than vehicle-treated rats when re-exposed to the drug-paired environment. Finally, the analysis of 'trill' 50-kHz USVs revealed significant effects only for amphetamine. This study further demonstrates that psychoactive drugs differ in their effects on 50-kHz USVs, and may help to understand the relevance and usefulness of these vocalizations in the study of the effects of drugs of abuse.

Drug-stimulated USVs

By showing that amphetamine was the only drug to significantly stimulate 50-kHz USVs during repeated administration and challenge, this study extends previous data on acute drug effects (Sadananda et al., 2012; Simola et al., 2012; Wright et al., 2012), and demonstrates that

MDMA, morphine, and nicotine fail to elicit 50-kHz USVs, even after repeated administration. This finding might be explained by the different mechanisms of action of the drugs evaluated, with emphasis on the modulation of dopamine (DA) transmission. Previous investigations have shown that stimulation of DA receptors in the nucleus accumbens (NAc) shell is crucial for 50-kHz USVs emission (Burgdorf et al., 2001, 2007; Wintink and Brudzynski, 2001; Thompson et al., 2006). The drugs evaluated in this study may all increase DA in the NAc shell, but whereas amphetamine directly elevates DA levels, MDMA, morphine, and nicotine elicit a weaker effect, and act through indirect mechanisms (Di Chiara and Imperato, 1988; Cadoni et al., 2005; Kleijn et al., 2011). Moreover, it is noteworthy that, at least for morphine and nicotine, repeated drug administration has been found to weaken, rather than sensitize, DA release in the NAc shell (Cadoni and Di Chiara, 1999, 2000). Finally, it is known that neurotransmitters other than DA may modulate 50-kHz USVs emission (Fu and Brudzynski, 1994; Wright et al., 2012), and that the drugs evaluated in this study can interact with these neurotransmitters (Kalivas, 2007). Therefore, the discrepancies in drug-stimulated 50-kHz USVs observed in this study could be explained by the different modulation of both DA and non-dopaminergic neurotransmitters by the drugs evaluated, although other mechanisms cannot be ruled out (see Simola et al., 2012 for a discussion).

Based on the hypothesis that 50-kHz USVs may indicate hedonia and positive affect (Knutson et al., 2002; Burgdorf et al., 2011), and considering that drugs of abuse target reward circuits and produce sensitization to some of their effects (Vanderschuren and Kalivas, 2000; Di Chiara et al., 2004), a progressive increase in 50-kHz USVs during the drug treatments in this study would have been expected. In line with earlier investigations (Hamed et al., 2012; Wright et al., 2012), this effect was observed for morphine, as the emission of 50-kHz USVs was greater on the fifth than on the first drug administration. However, rather than sensitization, this finding indicates tolerance to morphine-induced inhibition of 50-kHz USVs (Simola et al., 2012; Wright et al., 2012), as the number of vocalizations recorded on the fifth drug administration was similar to that of vehicle-treated rats. Similar, but not significant, modifications in 50-kHz USVs emission were observed within nicotine-treated rats, whereas rats administered MDMA on alternate days displayed a trend towards an increase in vocalization upon drug challenge.

To further clarify the significance of drug-stimulated 50-kHz USVs, it is interesting to compare these results with those of amphetamine-treated rats. Thus, amphetamine robustly stimulated 50-kHz USVs, but no modifications in the total number of vocalizations emitted were observed during repeated drug administration and challenge. Interestingly, this finding is in line with recent data showing that rats repeatedly treated with amphetamine did not substantially develop a sensitized 50-kHz USVs emission, and that no sensitization of 50-kHz USVs occurred in rats that self-administered methamphetamine (Taracha et al., 2012; Mahler et al., 2013). However, it is noteworthy that time-course analysis of amphetamine-stimulated 50-kHz USVs revealed a shift in the peak effect over drug treatment and challenge, which appears in line with earlier studies showing a sensitized emission of 50-kHz USVs following repeated administration of psychostimulants (Ahrens et al., 2009; Mu et al., 2009). Taken together, the results of the present study may suggest that, even though the evidence linking 50-kHz USVs with positive affect and reward is rather solid, the 50-kHz USVs observed after the administration of a drug could indicate not only drug-mediated reward, but also other effects. It can be hypothesized that drug-stimulated 50-kHz USVs may reflect, to some extent, behavioral activation and/or hyperstimulation of the dopaminergic system. This view would fit with data from the present and previous studies showing that dopaminergic drugs are most effective in eliciting 50-kHz USVs (Mu et al., 2009; Williams and Undieh, 2010; Wright et al., 2010, 2012; Brudzynski et al., 2012; Simola et al., 2012). In this regard, it is noteworthy that, even though DA is critical for the reinforcing properties of drugs (Di Chiara and Bassareo, 2007), elevations in DA levels, which may trigger 50-kHz USVs (Thompson et al., 2006), may not necessarily be indicative of a drug-induced positive affective state (Berridge and Robinson, 1998; Ikemoto, 2010).

USVs emitted on re-exposure to the test cage

A major finding of this study is that treatment with drugs that fail to directly stimulate 50-kHz USVs may induce long-lasting vocalizations upon re-exposure to the environment where the drug was administered. Thus, when rats were re-exposed to the test cage, 50-kHz USVs were emitted not only by amphetamine-treated rats, which vocalized following drug administrations, but also by rats that received MDMA or morphine, which did not. A possible explanation of these vocalizations may rely in the conditioning effects of the drugs evaluated. This is suggested by the finding that rats treated with morphine in the home cage, alternated with vehicle in the test cage, scarcely vocalized on test cage re-exposure. Further support to this view comes from the evidence that drug-treated rats displayed a higher spontaneous locomotor activity than vehicle-treated rats when re-exposed to the test cage, which could indicate drug-induced environmental conditioning. Interestingly, morphine-treated rats displayed a most pronounced and persistent 50-kHz USVs emission following test cage re-exposure, as this effect was still detectable 7 d after the last drug challenge, which appears in line with the ability of opiates to induce persistent conditioned responses (Nielsen and Kreek, 2012). At variance with

this, when re-exposed to the test cage, nicotine-treated rats exhibited a 50-kHz USVs emission not significantly different from that of vehicle-treated rats, likely attributable to a marked interindividual variability in this effect within and between the experimental groups.

The vocalization by amphetamine-treated rats re-exposed to the drug-paired environment observed in this study is in line with previous evidence (Knutson et al., 1999). On the other hand, some earlier studies, but not others, have reported conditioned 50-kHz USVs in morphine-treated rats re-exposed to a drug-paired environment (Knutson et al., 1999; Hamed et al., 2012; Wright et al., 2012). These discrepancies could be attributable to confounding factors, such as interindividual variability in vocalization rate, and differences in rat strain and drug-administration protocols. The present study evaluates, for the first time, the 50-kHz USVs emissions induced by different drugs of abuse under the same experimental conditions, and allows direct comparison of the effects of these drugs. Therefore, the present results suggest that 50-kHz USVs may be a valuable tool to investigate the long-lasting and conditioning effects of drugs, as they provide new evidence supporting the ability of amphetamine and morphine to elicit 50-kHz USVs following re-exposure to a drug-paired environment, and extend this finding to MDMA.

'Trill' USVs emission

Previous investigations have suggested that the 'trill' subtype of 50-kHz USVs may be a selective indicator of positive affect, as a sustained emission of these vocalizations has been reported in rats exposed to pleasurable stimuli, such as homospecific and heterospecific social contacts (tickling), or amphetamine administration (Burgdorf et al., 2008; Ahrens et al., 2009; Wright et al., 2010). In the present study, amphetamine was the only drug that elicited a significant emission of 'trill' vocalizations. Interestingly, the number of 'trill' 50-kHz USVs emitted did not undergo sensitization, but rather decreased during repeated amphetamine treatment. This seems in contrast with a previous study that observed sensitization of 'trill' 50-kHz USVs in rats repeatedly administered amphetamine (Ahrens et al., 2009). Again, this discrepancy is likely attributable to methodological issues, as the rat strain, the duration of the recording time, and the criteria used to classify 'trill' 50-kHz USVs. Nevertheless, and most importantly, the present results are in agreement with other studies that failed to observe a sensitized 'trill' 50-kHz USVs emission during either acquisition of morphine-induced conditioned place preference or methamphetamine self-administration (Wright et al., 2012; Mahler et al., 2013). The lack of a sensitized 'trill' 50-kHz USVs emission by amphetamine-treated rats might challenge the hypothesis that these vocalizations are selectively linked to hedonia. However, it is conceivable that, similar to observations in this study for the total 50-kHz USVs, the presence/absence of 'trill' vocalizations after the administration of a drug may indicate effects not necessarily, or exclusively, related to the hedonic properties of the drug. It is noteworthy that this study observed 'trill' 50-kHz USVs upon test cage re-exposure in all but nicotine-treated rats, although changes involving these vocalizations never reached significance.

Role of the administration protocol on USVs emission

The present study observed a similar pattern of drug-stimulated 50-kHz USVs between consecutive- and alternate-day administration, and this effect was evident for all the drugs evaluated. On the other hand, rats on alternate-day administration displayed a more marked vocalization when re-exposed to the test cage after drug discontinuation. Previous studies have suggested that the emission of 50-kHz USVs on re-exposure to a drug paired environment may indicate drug expectation in rats (Knutson et al., 2002; Ma et al., 2010; Maier et al., 2010). Moreover, experiments with cocaine have shown that drug expectation is increased in rats subjected to an intermittent protocol of substance administration (Puig et al., 2012), and that rats self-administering cocaine displayed a boost in the emission of 50-kHz USVs when re-exposed to cocaine-paired cues after a weekend of drug abstinence (Maier et al., 2010). Therefore, it can be hypothesized that rats on alternate-day treatment may experience a more pronounced drug expectation than rats on consecutive-day treatment. This, in turn, could justify why the former rats displayed a more marked 50-kHz USVs emission upon test cage re-exposure. It is noteworthy that this effect appeared to be long-lasting in rats treated with morphine, and that opiates are known to induce intense craving, which can persist long after drug discontinuation (NIDA, 2005). Therefore, the results of the present study may suggest that the intensity and/or resistance to extinction of 50-kHz USVs emitted on re-exposure to a drug-paired environment may be linked to drug craving, and that these vocalizations could be useful for studying drug seeking. However, further and more detailed investigations of the factors (e.g. drug dose and duration of administration) and of the drug-paired cues (e.g. environmental *vs.* introceptive) that are able to promote vocalizations are warranted to validate this hypothesis.

Conclusions

By evaluating different psychoactive drugs under comparable conditions, and so avoiding confounding factors related to the experimental procedure, the present study extends our previous findings and demonstrates that MDMA, morphine, and nicotine fail to stimulate 50-kHz USVs, even after repeated administration. Moreover, this study shows that drugs may have different direct and long-lasting effects on 50-kHz USVs. In fact, drugs that do not elicit 50-kHz USVs directly after administration,

such as morphine and MDMA, are nevertheless able to induce vocalizations in rats that are re-exposed to an environment paired with drug administration, which are likely dependent on the drugs' conditioning properties. Based on the results obtained, we suggest that 50-kHz USVs can be useful to evaluate the long-lasting effects and conditioning properties of psychoactive drugs, rather than their direct acute effects.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145713001235

Acknowledgments

This study was supported by funds from Regione Autonoma della Sardegna (Legge Regionale 7 Agosto 2007, N.7, annualità 2010) and from Fondazione Banco di Sardegna (project number 2013.1321). Dr Nicola Simola gratefully acknowledges Sardinia Regional Government for financial support (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007–2013 – Axis IV Human Resources, Objective l.3, Line of Activity l.3.1 'Avviso di chiamata per il finanziamento di Assegni di Ricerca'). Dr Lucia Frau gratefully acknowledges Sardinia Regional Government for financial support (Legge Regionale 7 Agosto 2007, N.7, annualità 2010, Attivazione Contratto Giovani Ricercatori, Contract 150/2013). The authors are grateful to Mr Renato Mascia, Department of Life and Environmental Sciences, University of Cagliari, for his help with the synthesis of MDMA. The authors acknowledge that the manuscript was edited for language and style by Medical Edit, Hoorn, The Netherlands, and that the authors are entirely responsible for the scientific content of the paper.

Statement of Interest

None.

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