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Abstract

Ultrasonic vocalizations are widely used to examine affective states in rats, yet relatively few studies explore the acoustic features of vocalizations, especially in relation to drug exposure, and no studies have explored alterations in acoustic features over time. The goal of this study was to examine nicotine- and phencyclidine-induced alterations of bandwidth, duration, and frequency of 50 kHz vocalizations. The minimum and maximum frequency, bandwidth, and duration of calls were examined after 7 days of daily subcutaneous administration of phencyclidine (2.0 mg/kg) and nicotine (0.2 and 0.4 mg/kg) in male Sprague-Dawley rats. Bandwidth was significantly decreased in rats treated with both nicotine (0.2 and 0.4 mg/kg) and phencyclidine. Maximum frequency was lowest on the first day of exposure compared with all other days and was not altered by drug exposure. Call duration was not affected by time or drug exposure. These findings suggest the importance of studying alterations in acoustic features in time, especially those induced by drug exposure.

Keywords: acoustic features, nicotine, phencyclidine, rat, ultrasonic vocalizations

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Introduction

Ultrasonic vocalizations (USVs) are widely considered a parameter for emotional states in adult rats (Knutson et al., 2002; Brudzynski, 2005, 2007; Panksepp, 2005; Schwarting et al., 2007) and can be divided into two main categories based on a general frequency range: 22 and 50 kHz. Although 22-kHz USVs are associated with negative or distressing situations (Litvin et al., 2007), 50-kHz USVs are typically emitted in response to appetitive contexts and pleasurable stimuli (Knutson et al., 1999; Burgdorf et al., 2000; Brudzynski, 2005). USVs in the 50 kHz range have also been utilized as a measure of drug reward (Thompson et al., 2006; Ahrens et al., 2009; Mu et al., 2009; Williams and Undieh, 2010).

Myriad psychoactive drugs with addictive and rewarding properties have acutely produced alterations in total 50-kHz USVs as well as specific subtypes of vocalizations, including amphetamine, cocaine, and methylphenidate (Williams and Undieh, 2010; Wright et al., 2010; Simola et al., 2012, 2014), whereas other drugs do not produce differences in total 50 kHz vocalizations. such as morphine, 3,4-methylenedioxymethamphetamine, and nicotine (Wright et al., 2010; Sadananda et al., 2012; Simola et al., 2010; 2014; Swalve et al., 2016). Vocalization patterns are also known to change over time, as seen with amphetamine and nicotine (Simola et al., 2014; Swalve et al., 2016). Acoustic features of 50 kHz vocalizations such as maximum frequency and bandwidth have recently been identified as a method for further examination of psychoactive substances and may have behavioral significance (Brudzynski, 2005; Simola et al., 2012), potentially encoding such information as magnitude or urgency of the signal, but few studies have examined these features after drug administration (Simola et al., 2012). Stimulants such as methylphenidate and nicotine differentially affect the acoustic features of 50 kHz vocalizations, with methylphenidate increasing the maximum frequency and bandwidth, whereas nicotine only increased bandwidth, suggesting that class and type of stimulant differentially affects acoustic features, yet sensitivity of these features to chronic use of drugs is currently unknown.

This study examined the long-term alterations in acoustic features of 50 kHz vocalizations produced by two drugs of abuse, phencyclidine (PCP) and nicotine. In a previous study, we found that chronic but not acute nicotine increased 50 kHz vocalizations, whereas PCP acutely

decreased the total number of vocalizations (Swalve et al., 2016). However, the long-term effects of nicotine on features such as bandwidth and maximum frequency as well as the potential significance of these chronic alterations are unknown. We hypothesized that nicotine would increase the maximum frequency and bandwidth similar to Simola et al. (2012), with frequency increasing over time. However, as the acoustic features of vocalizations during PCP exposure have never been examined, the direction of its effect cannot be predicted.

Methods

Subjects

Twenty-four male Sprague-Dawley rats (age 7–9 weeks, 275–299 g upon arrival) from Harlan Inc. (Indianapolis, Indiana, USA) were used. All rats were single-housed in clear rectangular polycarbonate tubs (48.3 cm × 26.7 cm × 20.3 cm) after arrival to avoid isolation-induced stress and vocalizations that would occur during the experimental procedures if rats were pair-housed. This dataset was analyzed for a prior experiment (Swalve et al., 2016) but all data reported here are new and have never been reported before. Rats had at least 5 days of habituation to the animal facility with 5 min of handling per day before experimental sessions commenced. Experiments were conducted during the light portion of a 12-h light/dark cycle (lights on from 6: a.m. to 6: p.m.). Housing facilities were maintained at ~22°C with relative humidity of 45–60%. Food and water were both freely available *ad libitum* throughout all phases of the experiment. All experiments were approved by the University of Nebraska–Lincoln Institutional Animal Care and Use Committee.

Apparatus

Eight identical two-way shuttle boxes, custom designed and manufactured by Med Associates (St Albans, Vermont, USA), housed in ventilated, sound-insulated isolation cubicles (96.52 cm width × 35.56 cm diameter × 63.5 cm height), were used as chambers. Each shuttle box was (64 cm × 30 cm × 24 cm) divided into two equal-sized compartments by a white PVC partition with a doorway (15 cm × 9 cm) with

an aluminum hurdle between the two compartments and a grid floor. A houselight (50 V) was located at the top of each chamber. All experimental sessions were controlled by Med Associates programs. Background noise (~74 dB) and ventilation was provided by a fan affixed outside of the chamber in the sound-attenuating cubicle. An USV microphone (P48 Avisoft Bioacoustics/Emkay Microphone; Avisoft Bioacoustics, Berlin, Germany) was mounted on the ceiling of experimental chamber, connected by an E-MU 0404 USB Audio device to a computer. Acoustic data were displayed in real time by the Avisoft RECORDER, a multichannel triggering hard-disk recording software (version 3.4; Avisoft Bioacoustics). Data were recorded at a sampling rate of 192 kHz in 16-bit format (version 4.51; Avisoft Bioacoustics).

Procedure

Each rat was habituated to the chambers for 30 min per day on 2 concurrent days. Pre-injections of nicotine were given to each rat 2 h after each habituation day in their home cages to limit the initial aversive effect of nicotine (Bevins and Besheer, 2001). Vocalizations of these animals had previously been used to examine the interaction of nicotine and PCP on total and categorized vocalizations (Swalve et al., 2016). In this experiment, four groups of rats were examined, divided into groups based on drug administration: SAL, SAL–NIC 0.2 mg/kg (NIC2), SAL–NIC 0.4 mg/kg (NIC4), and PCP. Over 7 total days, rats were injected with their corresponding drugs and placed into the chambers for 30 min, during which USVs were recorded.

Spectrograms were generated from the sound files using RavenPro 1.5 (Cornell Laboratory of Ornithology, Ithaca New York: 512 sample Fourier transformation with a Hann window function; filter bandwidth 135 Hz; frequency resolution 93.8 Hz; grid time resolution 2.13 ms). Each spectrogram was visually inspected by two separate trained scorers with an inter-rater reliability of 0.94. The scorers isolated each vocalization from the background noise and the acoustic features (minimum and maximum frequency, and duration) were calculated using the automatic measurement feature in RavenPro. Bandwidth was calculated by taking the maximum frequency and subtracting the minimum frequency for each call.

Drugs

(−) Nicotine tartrate salt (0.2 mg/kg or 0.4 mg/kg; Sigma Aldrich, St Louis, Missouri, USA) was dissolved in 0.9% saline to a final pH of 7.0 ± 0.2. Doses are expressed as base. Phencyclidine hydrochloride was obtained from the NIDA Chemical Synthesis and Drug Supply Program (Bethesda, Maryland, USA) and was mixed with 0.9% saline. All drugs were administered subcutaneously before placement in the chamber, with PCP administered 10 min and nicotine administered 5 min before placement. Two injections were given with at least one injection consisting of saline due to parameters established for Swalve et al. (2016).

Statistical analyses

Owing to unequal call numbers elicited by each subject, subjects with a minimum of 10 calls each day were selected and the means of each acoustic feature were used in all subsequent analyses (see **Table 1** for number of subjects and calls included; see Swalve et al., 2016 for analysis of number of calls). Seven rats were excluded due to this criteria (one from NIC2, one from NIC4, three from PCP, and two from saline only). Only 50 kHz calls (range: 35–95 kHz) were examined for this analysis given the low numbers of 22 kHz calls. Data across 7 days from the two nicotine (0.2 and 0.4 mg/kg) administrations were compared with the saline only administration using a mixed-model analysis of variance (ANOVA; drug × time) for each acoustic feature, followed by Fisher's least significant difference pairwise comparisons. Data across the 7 days from the PCP administration were compared

Table 1. Number of subjects in each condition and the average number of calls recorded per day of administration

		Average number of 50 kHz vocalizations						
Drug	*Number of subjects*	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*
SAL	6	602.83	272.00	190.17	189.00	189.33	173.00	116.33
NIC2	7	303.71	278.14	211.57	208.86	221.00	242.86	192.57
NIC4	6	218.50	247.67	191.33	226.17	187.17	216.33	172.17
PCP	5	223.80	178.40	242.80	253.00	234.20	223.40	227.20

NIC2, saline–nicotine (0.2 mg/kg); NIC4, saline–nicotine (0.4 mg/kg); PCP, phencyclidine; SAL, saline.

with the saline only condition using mixed-model ANOVAs (drug × time) for each acoustic feature, followed by Fisher's least significant difference pairwise comparisons. The criterion for statistical significance was P value less than 0.05.

Results

Nicotine

For nicotine administration, repeated-measures ANOVAs examining the acoustic features (duration, bandwidth, and maximum frequency) of the 50 kHz vocalizations found significant effects on the frequency measures but no significant effects of drug, time, nor interactions on the duration of calls (SAL: M = 0.049, SE = 0.001; NIC2: M = 0.046, SE = 0.001; NIC4: M = 0.046, SE = 0.001; NS). There was no significant main effect of drug nor interaction between drug and time on maximum frequency of 50 kHz calls. There were, however, significant changes in the maximum frequency across the 7 days of administration regardless of drug-type/dose [$F(6, 96) = 2.82$, $P < 0.02$, $\eta 2 = 0.15$] with significantly lower maximum frequency for calls emitted on day 1 compared with subsequent days ($P < 0.01$; **Fig. 1**). Follow-up repeated-measures ANOVAs, examining maximum frequency changes in 50 kHz subcategories across time, found that downward ramp, inverted-U, step down, and trill calls had significantly lower maximum frequency on day 1 ($P < 0.05$).

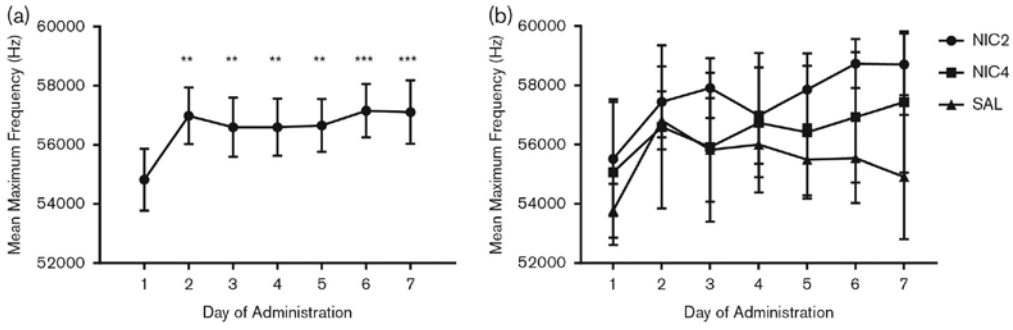

Figure 1. Rat 50 kHz vocalizations had a lower (a) maximum frequency (Hz) on day 1 of administration (***P*<0.01, ****P*<0.001) but there were no differences (b) in maximum frequency (Hz) between the drug treatments (*P*>0.05). NIC2, saline–nicotine (0.2 mg/kg); NIC4, saline–nicotine (0.4 mg/kg); SAL, saline.

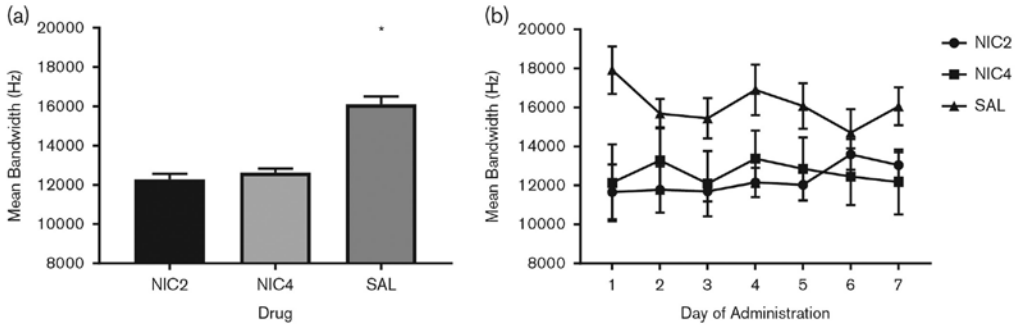

Figure 2. Mean bandwidth (Hz) during nicotine administration (a) was smaller in both NIC groups compared with saline (*P<0.05) but (b) did not change across the 7 days of treatment. NIC2, saline–nicotine (0.2 mg/kg); NIC4, saline–nicotine (0.4 mg/kg); SAL, saline.

A repeated-measures ANOVA found significant changes in the bandwidth of the 50 kHz calls during nicotine administration. There was a significant main effect of drug [$F(2, 16) = 3.83$, $P < 0.05$, $\eta^2 = 0.32$; **Fig. 2**a]. Pairwise comparisons showed that bandwidth was significantly smaller for rats administered NIC2 (Hz; M = 12285.62, SE = 1018.90, P <0.025) and NIC4 (Hz; M = 12625.85, SE = 1100.54, P < 0.05) compared with those administered saline (M = 16110.57, SE = 1100.54) but there was no significant difference between the two nicotine doses. There was no significant effect of time nor interaction between drug and time on the bandwidth of 50 kHz calls, although the interaction was trending towards significance (P = 0.052; Fig. 2b). Follow-up one-way ANOVAs, examining the subcategories of 50-kHz USVs, determined that complex calls decreased in bandwidth following low dose nicotine administration (P <0.05), and downward ramp and multistep calls had decreased bandwidth after high dose nicotine administration compared with saline (P < 0.05; **Table 2**).

Phencyclidine

For PCP administration, repeated-measures ANOVAs examining the acoustic features of the 50 kHz vocalizations (duration, bandwidth, and maximum frequency) showed that PCP and length of administration led to changes in frequency measures (Hz) but there were no significant changes in call duration between PCP (M = 0.050, SE = 0.002) and SAL (M = 0.049, SE = 0.002) administration or across administration days.

Table 2. Average bandwidth (kHz) and SDs for each 50-kHz ultrasonic vocalization subcategory

	Bandwidth [mean (SD)]			
Category	*SAL*	*NIC2*	*NIC4*	*PCP*
Complex	10.93 (1.75)	8.87 (0.69)*	9.09 (2.11)	9.60 (2.04)
Composite	32.68 (3.92)	28.86 (–)	–	28.78 (2.23)
Downward ramp	10.50 (2.99)	7.72 (0.70)	6.93 (1.30)*	5.81 (0.77)*
Flat	2.88 (0.81)	2.60 (0.13)	2.75 (–)	2.37 (0.88)
Flat trill	–	–	–	–
Inverted-U	10.08 (3.90)	9.64 (0.76)	8.29 (0.57)	9.42 (0.51)
Multistep	27.27 (2.56)	23.02 (0.94)	20.56 (4.20)*	29.25 (6.79)
Short	4.85 (–)	3.95 (0.15)	–	3.53 (–)
Split	42.15 (1.89)	39.69 (–)	–	37.46 (–)
Step down	22.58 (4.45)	19.77 (0.02)	–	15.45 (–)
Step up	21.87 (3.65)	21.34 (2.27)	20.46 (2.01)	23.20 (2.92)
Trill	14.00 (3.95)	10.38 (0.75)	11.29 (4.76)	12.81 (3.63)
Trill with jumps	28.91 (3.72)	25.70 (1.38)	26.55 (0.61)	29.17 (3.37)
Upward ramp	9.52 (2.41)	8.75 (1.05)	9.04 (3.18)	8.37 (1.84)

NIC2, saline–nicotine (0.2 mg/kg); NIC4, saline–nicotine (0.4 mg/kg); PCP, phencyclidine; SAL, saline.

*P <0.05, significant difference compared with saline administration.

There was no significant main effect of drug nor interaction between drug and time on maximum frequency of 50 kHz calls emitted by rats. However, there were significant changes in the maximum frequency across the 7 days of administration regardless of drug-type [$F(6, 54) = 2.67$, $P<0.05$]. Pairwise comparisons revealed that calls emitted on day 1 of administration had significantly lower maximum frequency than all subsequent days ($P<0.05$; **Fig. 3**). Follow-up repeated-measures ANOVAs, examining maximum frequency changes in 50 kHz subcategories across time, found that flat and step-up calls had significantly lower maximum frequency on day 1 ($P<0.05$).

Bandwidth, in contrast, was affected by drug administration. Rats receiving PCP treatment had significantly smaller bandwidth (Hz; $M = 12411.18$, $SE = 1054.37$) compared with those administered saline ($M = 16110.57$, $SE = 962.50$) [$F(1, 9) = 6.72$, $P<0.05$; **Fig. 4**a]. There was no significant effect of time on bandwidth, and no significant drug x time interaction; though pairwise comparisons between drug-type on each day of administration, showed that PCP only had significantly smaller bandwidth on days 1, 4, and 5 ($P<0.05$; Fig. 4b). Follow-up one-way ANOVAs, examining the subcategories of 50-kHz

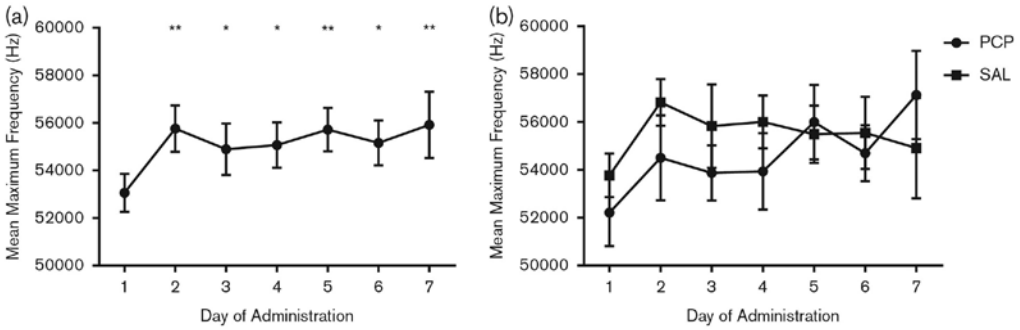

Figure 3. Rat 50 kHz vocalizations had a lower (a) maximum frequency (Hz) on day 1 of administration (*P<0.05, **P<0.01), but there was no difference (b) between the PCP and saline treatment groups. PCP, phencyclidine; SAL, saline.

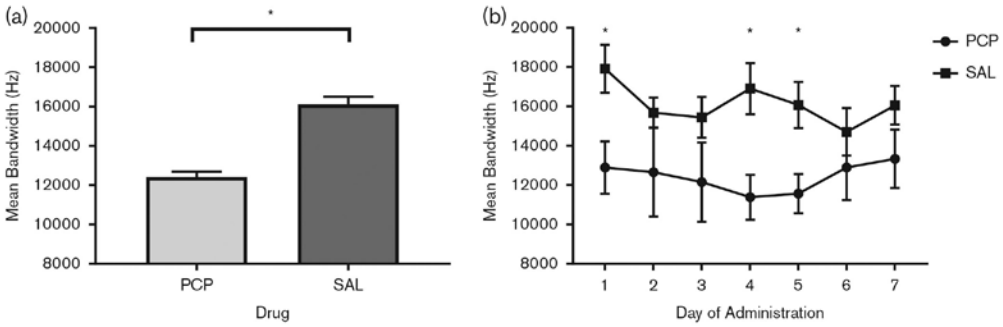

Figure 4. PCP administration (a) decreased the bandwidth of 50 kHz vocalizations compared with saline administration (*P< 0.05) and (b) resulted in change across the 7 days of administration. PCP, phencyclidine; SAL, saline.

USVs, determined that only downward ramp calls had significantly decreased bandwidth after PCP administration compared with saline (P<0.05; Table 2).

Discussion

This study examined the alteration in acoustic features of 50-kHz USVs induced by nicotine and PCP exposure. Nicotine did not affect the duration or maximum frequency of calls but decreased bandwidth. PCP administration led to changes in frequency measures, including a decrease in bandwidth compared with saline. In addition, there was a general trend of increasing maximum frequency levels over days for

all calls, not dependent on the drug group. This study is the first to show that PCP alters the acoustic features of USVs over time.

Both a low and a high dose of nicotine (0.2 and 0.4 mg/kg) led to a decrease in bandwidth but did not alter other features such as call duration or maximum frequency. In addition, bandwidth decreases in complex, downward ramp, and multistep calls contributed to this overall decrease after nicotine administration. This finding is somewhat in opposition to the study of Simola et al. (2012). Although the finding that a low dose of nicotine (e.g. 0.2 mg/kg) was not enough to evoke differences in frequency measures is similar to that of Simola et al. (2012), they found an increase in both maximum frequency and bandwidth with the high dose (e.g. 0.4 mg/ kg) and no differences in bandwidth of the 50-kHz USV subcategories.

The lack of modification of maximum frequency and contradictory alterations in bandwidth between the current findings and Simola et al. (2012) could be explained by methodological differences. In Simola et al. (2012), recording sessions lasted 2 h, compared with the 30min sessions used here. One potential explanation for this difference is that a decrease in the total number of vocalizations due to the shortened testing length might have led to low levels of vocalizations, during which differences in certain features such as maximum frequency could have been masked due to a lack of data points. However, even within a shortened session, a minimum of 10 calls were examined, with an average of 211 calls, higher than those previously reported in Simola et al. (2012), suggesting that such an effect was not likely and was not seen in our study. Indeed, other differences in the methodology between this work and Simola et al. (2012), such as chamber composition, latency to start recording, and total days of acclimation, could potentially explain differences between their findings and ours. Given the contrast in findings, future research on the short-term and long-term effects of nicotine on acoustic features of vocalizations is strongly recommended, including an exploration of parametric work on the environment most likely to produce vocalization differences.

Similar to that seen with nicotine, PCP exposure led to smaller bandwidth compared with saline groups. Upon further examination of subcategories, PCP administration reduced bandwidth in downward ramp calls. The specific modification of bandwidth in comparison to other acoustic features suggests that this feature might relate to certain aspects of communication. This literature suggests that

vocalizations can be subdivided into 22 and 50 kHz in relation to affective states (Knutson et al., 1999; Burgdorf et al., 2000; Brudzynski, 2005; Litvin et al., 2007). However, it is possible that alterations of bandwidth may also be implicated in affective state. Indeed, bandwidth and other acoustic features have been purported to play a significant role in communication (Brudzynski, 2005) and may 'convey physiological states' (Stewart et al., 2015), as decreased bandwidth was associated with increased heart rate, which may be related to distress. The role of bandwidth in communication is comparatively unknown and its relation to the reinforcing value of drugs is unknown. Nicotine is purportedly reinforcing and increased bandwidth in Simola et al. (2012), along with other drugs that induce rewarding states such as morphine and methylphenidate, but decreased bandwidth in this study. PCP, in direct contrast, produces a decrease in rewarding states (Spielewoy and Markou, 2003) and decreased bandwidth in this study. In addition, both D1 and D2 antagonists significantly decrease bandwidth (Ringel et al., 2013), and dopamine is linked to many aspects of continued drug use including motivation and reward-related stimuli (Berridge and Robinson, 1998). Thus, alterations in bandwidth may be related to properties of drugs other than reinforcement, which should be examined in future studies.

Neither PCP nor nicotine altered maximum frequency, although rats in all groups showed an increase in maximum frequency over days. This increase in maximum frequency was primarily driven by low maximum frequency on the first day of testing in all groups. It may be possible that experimental parameters could be the cause of low maximum frequency on the first day, such as the potential stressor of the injections; however, animals in the nicotine group had previously undergone injections in their home cage so any alterations in vocalizations due to injections should have been diminished in at least one of the four groups. Although this acoustic feature may not be integral in examining the effects of drugs, determining how vocalizations change over time is important in future research on vocalizations. Maximum frequency was altered by nicotine administration in Simola et al. (2012) and this particular feature has been suggested to be of particular importance in communication (Brudzynski, 2005). Links between maximum or peak frequency and hedonic aspects of drugs have also been hypothesized, as dopamine antagonists differentially disrupted this measure (Ringel et al., 2013). Further examination of

drug-induced alterations of acoustic features over time is warranted, as well as elucidation of natural changes in bandwidth and frequency and whether these are affected by specific environmental conditions.

Chronic treatment with both PCP and nicotine significantly decreased bandwidth. Maximum frequency was not altered dependent on drug exposure but increased over time. These findings suggest that acoustic features of natural and drug-induced USVs may change over time and future research on USVs as a measure of affective states in rats should take this variable into account.

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Conflicts of interest — No conflicts of interest are reported.

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