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ORIGINAL INVESTIGATION

Effects of anxiogenic drugs on the emission of 22- and 50-kHz ultrasonic vocalizations in adult rats

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Abstract

Rationale Adult rat 22-kHz vocalizations are often associated with alarm or distress, whereas a subset of 50-kHz calls is preferentially emitted in response to amphetamine and other rewarding stimuli. Whether any 50-kHz calls reflect anxiety is unknown.

Objective To determine the effects of anxiogenic drugs on 50-kHz call rate and call subtype profile, in comparison with D-amphetamine.

Methods Adult male rats received systemic amphetamine (1 mg/kg) three times several days before testing. Ultrasonic vocalizations were then recorded after acute intraperitoneal injection of amphetamine or one of five anxiogenic drugs: yohimbine (2.5 mg/kg), *N*-methyl-β-carboline-3-carboxamide (FG 7142, 5 mg/kg), pentylenetetrazol (PTZ, 20 mg/kg), *m*-chlorophenylpiperazine (mCPP, 1 mg/kg), caffeine (25 mg/kg), or vehicle.

Results The duration of immobility was increased by FG 7142, PTZ, and mCPP; this measure was unchanged by yohimbine and reduced by the locomotor stimulant drugs amphetamine and caffeine. Conversely, the 50-kHz call rate was reduced by FG 7142, PTZ and mCPP, and increased by caffeine and amphetamine. Overall, the most common 50-kHz call subtypes were flat, trill, step-up, and complex. Consistent with previous reports, amphetamine increased the relative prevalence of trill calls while reducing the relative prevalence of flat calls. Yohimbine and caffeine reduced flat call prevalence, whereas mCPP reduced trill call prevalence. No other shifts in the call profile were observed, and no anxiogenic drug induced 22-kHz calls.

Conclusion Anxiogenic drugs, as a class, did not uniformly alter the 50-kHz call rate or subtype profile. Amphetamine-induced effects on 50-kHz call rate and profile do not reflect anxiety.

Keywords Ultrasonic vocalization · Anxiety · FG 7142 · *m*-Chlorophenylpiperazine (mCPP) · Anxiogenic · Amphetamine

Introduction

In the search for novel anxiety tests (Haller and Alicki 2012), rodent ultrasonic vocalizations (USVs) are of interest since they appear to convey information about emotional states (Brudzynski 2013; Burgdorf and Moskal 2010; Miczek et al. 1995; Wöhr and Schwarting 2010). Rats, in particular, emit USVs in three broad categories: infant 40 kHz, adult 22 kHz, and adult 50 kHz. To date, two of these three USV categories have been exploited as measures of anxiety: infant 40-kHz isolation calls that are triggered by maternal separation, and adult "distress/alarm" 22-kHz calls that are evoked by electric shock or shock-conditioned stimuli (Miczek et al. 1995; Sanchez 2003; Schwarting and Wöhr 2012; van der Poel and Miczek 1991).

Adult 50-kHz vocalizations, in contrast to 40- and 22-kHz calls, are spontaneously emitted in many behavioral contexts

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(Brudzynski 2013; Clarke and Wright 2014; Panksepp and Burgdorf 2010; Simola 2015; Wöhr and Schwarting 2010), but a possible association with anxiety has not been investigated extensively. The rate of 50-kHz call emission is reportedly inhibited by several anxiogenic conditions, including cat odor, repeated intermittent footshock, and bright illumination presented with or without an elevated platform (Ishiyama and Brecht 2016; Panksepp and Burgdorf 2010; Taylor et al. 2017). In contrast, 50-kHz call rate was decreased by neither yohimbine nor caffeine, even when given at doses that are typically anxiogenic (Mahler et al. 2013; Simola et al. 2016, Simola et al. 2010). A striking feature of 50-kHz calls is their high degree of heterogeneity, with 14 call subtypes identified to date (Wright et al. 2010), but it is unknown whether anxious rats preferentially emit any particular kind of 50-kHz call (Wright et al. 2010).

The main aim of the present study was therefore to establish whether one or more 50-kHz call subtypes, or possibly a change in the 50-kHz call rate, might serve as a marker for anxiety. To this end, we asked whether systemic administration of prototypical anxiogenic drugs produces a uniform and characteristic shift in the 50-kHz call profile. The five drugs selected for this purpose were yohimbine, FG 7142, pentylenetetrazol (PTZ), meta-chlorophenylpiperazine (mCPP), and caffeine. All five drugs are reported to be anxiogenic in human subjects and to produce anxiety-like behavior in rats, via diverse but incompletely identified receptor mechanisms (Table 1 and Supplementary Table 1). In particular, mCPP appears to act mostly via 5-HT2C receptor agonism (Gibson et al. 1994; Kennett et al. 1989), whereas FG 7142 and PTZ likely act via negative allosteric modulation of $GABA_A$ receptors (Evans and Lowry 2007; Huang et al. 2001). Mechanisms underlying yohimbine and caffeine anxiogenesis have yet to be identified, but it is unlikely that the main pharmacological targets of these drugs (α2 adrenergic receptors and adenosine receptors, respectively) play a significant role in rodents (Baldwin and File 1989; El Yacoubi et al. 2000; La Marca and Dunn 1994; Redfern and Williams 1995).

In the present study, we performed parallel tests with the psychostimulant D-amphetamine. This drug served in part as a positive control, since it reliably increases the 50-kHz call rate and predictably alters the call profile, i.e., the relative proportions of individual 50-kHz call subtypes (Wright et al. 2010). Specifically, amphetamine decreases the relative prevalence of the "flat" call subtype while promoting a narrowly defined "trill" call subtype (Wright et al. 2010). This particular call profile shift, which is also found with the euphorigens cocaine and morphine, has been proposed to reflect positive affect (Best et al. 2017; Pereira et al. 2014; Wright et al. 2012; Wright et al. 2010). In the present study, we tested the specificity of this claim, by investigating whether the same call profile shift is also associated with drug-induced anxiety.

Methods

Subjects

Subjects were 24 experimentally-naïve male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 310–360 g at the beginning of the experiment. Only male rats were used, for two reasons. First, all anxiogenic test drugs and doses have been extensively characterized in male but not female rats, with some evidence of sex differences either in baseline performance or anxiogenic effects of drugs (Haleem 1993; Hughes and Hancock 2016; Johnston and File 1991). Second, we wished to determine whether anxiogenic drugs would mimic the effects of amphetamine on 50-kHz call emission, which, to our knowledge, have been characterized in male rats only. The rats were housed three per cage (25 × 48 × 20 cm) in a temperature- and humidity-controlled colony room (19–20 °C, 50–60%) at the McGill University Animal Research Center. Home cage bedding consisted of laboratory-grade SaniChips™ (Harlan Laboratories, Indianapolis, IN). Rats were maintained on a reverse 12:12-h light/dark cycle with lights off at 0700 hours, and all testing was performed during the dark phase of the cycle. Food and water were available ad libitum except during testing. Rats were each handled once daily for 3 min, for 3 days prior to the habituation day. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Test box

The test box comprised four vertical walls enclosing a square arena (61 × 62.5 cm). The walls were 53 cm high and made of wood composite coated by white plastic laminate. The enclosed floor area was covered with a layer of bedding (SaniChips™, as in the home cage) which was replaced between test sessions. The test box was lit by far-red (wavelength > 650 nm) illumination provided by two 40-W incandescent lights, each in combination with a Kodak GBX-2 safelight filter (Vistek, Toronto, ON, Canada) located 1.4 m above the floor. A video tracking system (EthoVision version 3.0, Noldus Information Technology, Leesburg, VA, USA) measured locomotor activity (expressed as the total distance moved) and immobility duration. The latter measure represented the total accumulated time spent with the animal exhibiting zero horizontal movement, as detected by the imaging system (set at five frames/s). Immobility duration served to approximate freezing behavior and was previously found to be correlated with aversive 22-kHz USV emission (Wöhr and Schwarting 2008).

Acquisition and acoustic analysis of ultrasonic vocalizations

Broadband recordings and acoustic analysis were performed essentially as detailed in our recent publications (e.g., Scardochio and Clarke 2013). Rats were recorded individually via an ultrasonic condenser microphone that was positioned 50 cm above the center of the test box and connected to an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics). The sampling rate was 250-kHz with 16-bit resolution. Spectrograms were generated by fast Fourier transform (512 points, 75% overlap, FlatTop window, 100% frame size) using Avisoft SASLab Pro (Version 5.2.07). All calls in a given session were manually selected from spectrograms by one individual (M.W.) and then verified by another (L.M.B.). Time-sampling was not used, i.e., all calls were analyzed in each test session. Fifty-kilohertz calls were categorized according to our 14-subtype scheme (Wright et al. 2010). Twenty-two-kilohertz calls were also identified, but rarely occurred.

Drugs

Drugs were as follows: caffeine and D-amphetamine sulfate (both from Sigma-Aldrich, Oakville, ON); FG 7142 (*N*-methyl-β-carboline-3-carboxamide), pentylenetetrazol (PTZ, 6,7,8,9-tetrahydro-5*H*-tetrazolo[1,5-*a*]azepine), mCPP (m-chlorophenylpiperazine HCl), and yohimbine HCl (all from Tocris Bioscience, Minneapolis, MN). Drugs were dissolved in sterile 0.9% saline, with the following two exceptions. Yohimbine was dissolved in distilled water, as it was insufficiently soluble in saline. FG 7142 was prepared as a suspension in a mixture of 5% (*v*/*v*) Tween-20 and 0.9% saline, and sonicated for 5 min. Immediately after preparation, all drugs were divided into aliquots and stored at − 20 °C until the day of use. All drugs were administered by intraperitoneal (IP) injection in a volume of 1 ml/kg. The control for each drug condition was the corresponding vehicle. All doses refer to the salt of the compound (except caffeine). Each drug was tested at a single dose, as follows: yohimbine 2.5 mg/kg, FG 7142 5 mg/kg, PTZ 20 mg/kg, mCPP 1 mg/kg, caffeine 25 mg/kg, and amphetamine 1 mg/kg. Doses of the anxiogenic drugs were based on published studies of anxiety-like behaviors (see Supplementary Table 1 for references) and were intended to produce sub-maximal effects. All doses were sub-convulsive, as reported for yohimbine (Cole et al. 1995), FG 7142 (Leidenheimer and Schechter 1988), PTZ (Eidman et al. 1990), mCPP (Cioli et al. 1984), and caffeine (Chu 1981).

Experimental overview and protocol

The experiment comprised the following stages: habituation in the test room (one session), amphetamine pre-tests (three sessions), and the anxiogenic drug testing block (12 sessions). On any given day, rats were taken in their home cages to the test room and left to settle for at least 20 min.

Habituation (one test day) Each rat ($n = 24$) was individually placed in the test cage for 20 min before being returned to its home cage.

Amphetamine pre-tests (three test days) Each rat received three 10-min test sessions in the test box, one session per day, with sessions spaced 2 days apart. Each session started 30 min after amphetamine injection. The aim of these sessions was to increase 50-kHz call rates (Scardochio and Clarke 2013; Wright et al. 2013).

Drug testing block (12 test days) Each rat received 12 test sessions and was tested under all drug conditions, as follows: yohimbine 2.5 mg/kg, FG 7142 5 mg/kg, PTZ 20 mg/kg, mCPP 1 mg/kg, caffeine 25 mg/kg, and amphetamine 1 mg/kg. Control tests were performed under the respective vehicle condition, i.e., water (for yohimbine), Tween-20/saline (for FG 7142), or saline. Each drug and vehicle condition was tested once, except for saline (twice) and amphetamine (three times). Test days were spaced 48 h apart, and the order of drug and vehicle testing was determined by two 12 × 12 Williams squares, i.e., counterbalancing for first-order carryover effects. The experimenter (M.W.) was blinded to treatment conditions. On a given day, rats were tested individually in the test box, for 10 min starting 30 min after a single injection of a drug or vehicle. At the start of each session, rats were placed facing the same corner.

Data analysis and statistics

Data were analyzed using Systat v11 software (SPSS, Chicago, IL), and figures were generated using Prism 4 (GraphPad Software, La Jolla, CA). The main dependent variables analyzed were as follows: duration of immobility, distance moved, 50-kHz call rate, and the percentages of flat and trill calls (i.e., the two most prevalent 50-kHz call subtypes). In addition, 50-kHz call profiles were defined by the proportional contributions of all 14 call subtypes (Wright et al. 2010). The 50-kHz USV and locomotor data were consistent across the two saline tests and across the three amphetamine tests; hence, these data sets were collapsed across sessions. One rat received the incorrect treatment on several occasions, through a coding error, and was excluded from all analyses. Therefore, all results are reported for $n = 23$ rats, except where noted. Two outliers, identified by Grubb's test (two-tailed $p < 0.01$), were excluded from parametric analyses (specifically: rat #21 immobility under FG 7142, $z = 4.23$, and rat #6 percentage of flat calls under PTZ, $z = 3.03$).

Drug effects were analyzed by comparing each drug condition with its corresponding vehicle control (i.e., saline, water, or Tween-20). For this purpose, paired *t* tests were used,

provided that the parametric test assumptions were met. Otherwise, Wilcoxon signed-ranks tests were used, i.e., to assess the effect of yohimbine on percent flat calls, and to test effects of all drugs on call rate data.

Exploratory correlational analyses were employed to explore possible relationships between distance moved, immobility duration, 50-kHz call rate, percentage of flat calls, and percent of trill calls. Here, Pearson correlations were calculated (exception 50-kHz call rate, Spearman correlations). Before analysis, the effects of the six drugs were isolated by subtracting the corresponding vehicle (control) values. In most cases, the sample size (n) was 23 rats, but some drugs (mCPP, PTZ, FG 7142, yohimbine) eliminated calling in certain animals so that the percentage of flat and trill calls could not be calculated; in such cases, n ranged between 14 and 20 rats.

In view of the large number of statistical tests performed, the two-tailed significance threshold was set at 1% throughout.

Results

Table 1 summarizes the main findings.

22-kHz and 50-kHz call rates Only one rat made any 22-kHz vocalizations, and these occurred in a single session under amphetamine (41 calls). Total 50-kHz call rates (i.e., number emitted per 10-min session) are shown in Figs. 1 and 2a. This measure was increased by caffeine and amphetamine, and reduced by PTZ and mCPP (Wilcoxon tests $Z = 3.12$–4.14, $p = 0.0018$–0.0001), with a similar inhibitory trend for FG 7142 ($Z = 2.39$, $p = 0.0169$). Yohimbine did not detectably alter the 50-kHz call rate.

Flat and trill call subtypes The relative prevalence (percentage) of flat and trill calls is shown in Fig. 2b, c. As expected, amphetamine increased the percentage of trill calls ($t_{22} = 3.23$, $p = 0.0038$) at the expense of flat calls ($t_{22} = 2.79$, $p = 0.0106$). Flat calls were also suppressed by yohimbine (Wilcoxon $Z = 3.22$, $p = 0.0013$) and caffeine ($t_{22} = 3.08$, $p = 0.0054$), but unlike amphetamine, neither drug significantly promoted trill calls (yohimbine $p = 0.0685$, caffeine $p = 0.2609$). Trill calls were inhibited by mCPP ($t_{13} = 3.68$, $p = 0.0028$).

50-kHz call profiles The proportion of all 14 50-kHz call subtypes is shown in Fig. 3 (see Supplementary Tables 2 and 3 for *absolute* data). In virtually all treatment conditions, the most prevalent 50-kHz call subtypes were complex, flat, step-up, and trill. No call subtypes, other than flat and trill calls, were significantly affected by any drug.

Locomotor activity and immobility Locomotor activity (i.e., distance moved) was inhibited by PTZ and mCPP, and

Table 1 Summary of results in relation to published anxiety-related studies

	Yohimbine	FG 7142	PTZ	mCPP	Caffeine	Amphetamine
Dose tested (IP)	2.5 mg/kg	5 mg/kg	20 mg/kg	1 mg/kg	25 mg/kg	1 mg/kg
Present study						
22-kHz call rate	–	–	–	–	–	–
50-kHz call rate	–	↓?	↓	↓	↑	↑
% flat	↓	–	–	–	↓	↓?
% trill	–	–	–	↓	–	↑
Immobility duration	–	↑	↑	↑	↓?	↓
Horizontal LMA	–	–	↓	↓	↑	↑
Anxiety-related measures in rats (published studies)						
Freezing or immobility	↑	↑	n/a	n/a	↑?	Mixed
EPM (time in open arms)	↓	↓	↓	↓	↓	Mixed
Social interaction	Unchanged or ↓	↓	↓	↓	↓	Mixed
Other measures (published studies)						
Anxiogenic (humans)	Yes	Yes	Yes	Yes	Yes	Mixed
Horizontal LMA (rats)	Unchanged or ↓	Unchanged or ↓	↓	Unchanged or ↓	↑ or ↓	↑
CPA (rats)	CPA or CPP	CPA or no effect	CPP	No effect	CPA	CPP

Stimulatory and inhibitory drug effects are shown by ↑ and ↓ symbols, respectively. An added question mark denotes a non-significant trend, whereas an en dash (i.e., "–") indicates no trend. Findings are also shown from representative published studies which used the same or similar doses; the corresponding literature references are given in Supplementary Table 1. In the rat EPM and social interaction tests, anxiety-like behavior is shown as "↓"

n/a no information appears available at relevant doses, *PTZ* pentylenetetrazol, *IP* intraperitoneal, *LMA* locomotor activity, *EPM* elevated plus maze, *CPA/CPP* conditioned place aversion/preference

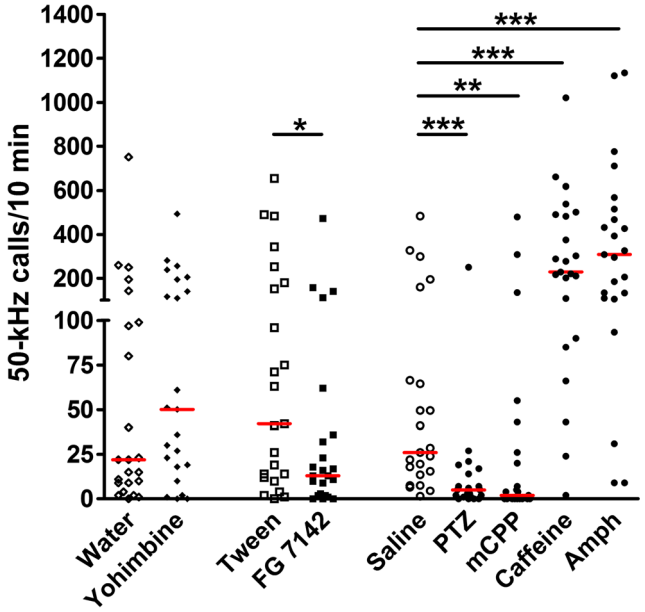

Fig. 1 Effects of anxiogenic drugs on the 50-kHz call rate. The *Y*-axis displays the number of calls made in the 10-min test session. Within a given drug condition, each data point represents an individual rat, and the horizontal bar shows the median. Each rat was tested under all drug conditions (see main text for details), and drugs are shown grouped with the respective vehicle condition, i.e., water, Tween-20/saline, or saline. Call rates were significantly increased by caffeine and amphetamine, and reduced by PTZ and mCPP. $*p = 0.02$ (i.e., trend), $**p < 0.01$, $***p < 0.001$ vs. vehicle control ($n = 23$ rats)

increased by caffeine and amphetamine ($t_{22} = 3.57–7.93$, $p = 0.0017–0.0001$; Fig. 2d). The corresponding percent changes were −25, −19, 18, and 35%, respectively, compared to vehicle control conditions. The duration of immobility was significantly increased by PTZ (by 88%, $t_{22} = 2.92$, $p = 0.0079$), mCPP (21%, $t_{22} = 2.98$, $p = 0.0070$), and FG 7142 (51%, $t_{21} = 3.18$, $p = 0.0045$), as shown in Fig. 2e. It was markedly *decreased* by amphetamine (by 70%, $t_{22} = 7.52$, $p = 0.0001$), with a similar trend for caffeine (35% decrease, $t_{22} = 2.81$, $p = 0.0101$), whereas yohimbine had no effect ($p = 0.6673$).

Correlational analyses Exploratory correlational analyses were used to explore possible relationships between the following variables: distance moved vs. immobility; percent flat vs. percent trill calls; and the two locomotor measures vs. 50-kHz call rate, percent flat, and percent trill. In this way, 48 correlation coefficients were generated (i.e., eight comparisons × six drugs), of which only four were statistically significant at the 1% level, as follows. Distance moved and immobility were negatively correlated ($p < 0.01–0.001$) with four individual drugs, i.e., yohimbine ($r = -0.7149$), PTZ ($r = -0.5886$), mCPP ($r = -0.5690$), and amphetamine ($r = -0.5698$), with similar trends ($p < 0.05$) for FG 7142 ($r = -0.4233$) and caffeine ($r = -0.4811$). The percentage of flat calls was not significantly correlated with

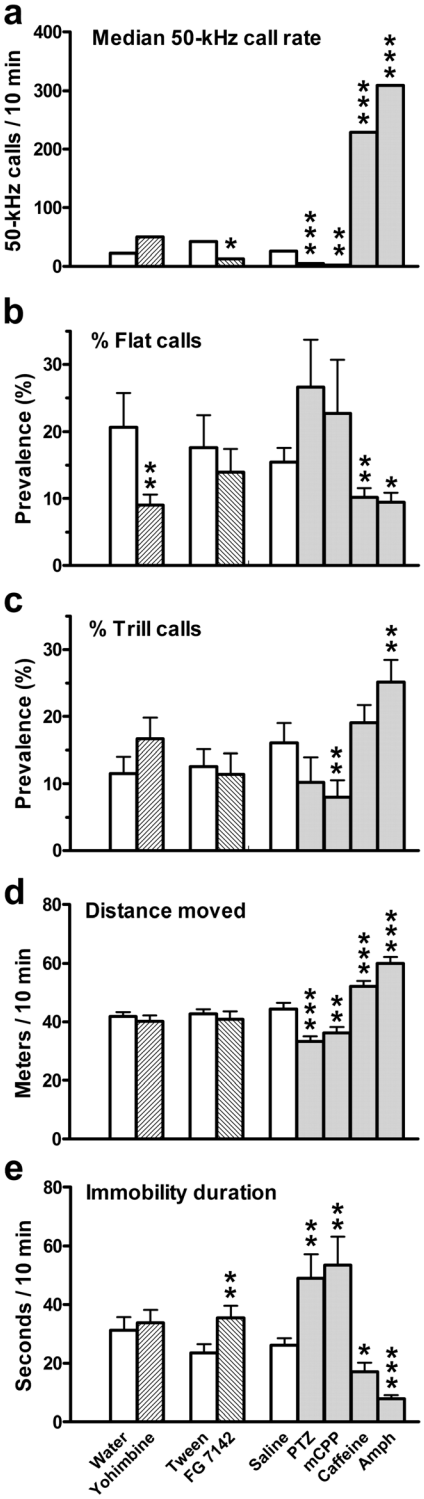

Fig. 2 Drug effects on 50-kHz vocalizations and video-based measures. *Y*-axes in panels **a**–**e** represent, respectively, median call rate (calls per 10-min test session), and mean ± SEM relative prevalence of flat and trill calls (expressed as a percentage of all 50-kHz calls), distance moved (m), and duration of immobility (s). Generally, $n = 23$ rats; however, calls were not emitted in certain individual sessions, hence for percentage flat and trill call measures, $n = 19–23$ rats except $n = 14$ rats for mCPP. $*p = 0.01$ (see text); $**p < 0.01$; $***p < 0.001$

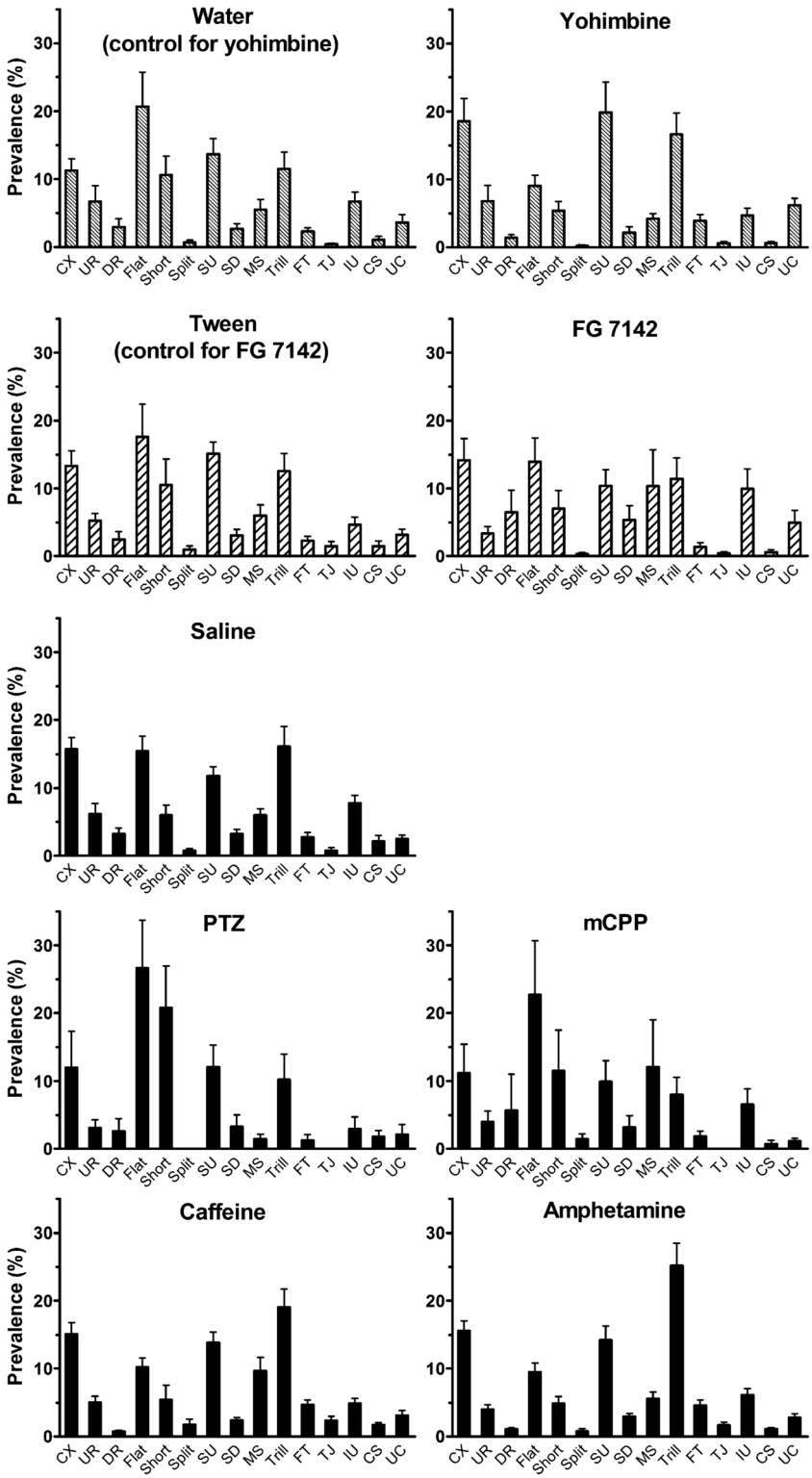

Fig. 3 Drug effects on 50-kHz call subtype profiles. *Y*-axes show the relative prevalence of each call subtype, expressed as the mean ± SEM percentage of all 50-kHz calls emitted. Call subtype abbreviations: CX complex, UR upward ramp, DR downward ramp, FL flat, SH short, SP split, SU step-up, SD step-down, MS multi-step, TR trill, FT flat-trill, TJ trill with jumps, IU inverted-U, CS composite, UC unclear. As explained in Fig. 2 legend, n = 19–23 rats in all conditions except for mCPP (n = 14). See Supplementary Tables 2 and 3 for absolute call rates

percent trill calls for any drug condition (r = − 0.4592 to + 0.1102). Similarly, neither of the two locomotor measures (distance moved and immobility duration) were significantly correlated with any of the three USV variables (r = − 0.4592 to + 0.5227). In particular, no significant correlation was found between the effects of any given drug on immobility and the 50-kHz call rate (Spearman rho = − 0.2743 to + 0.0845; Fig. 4).

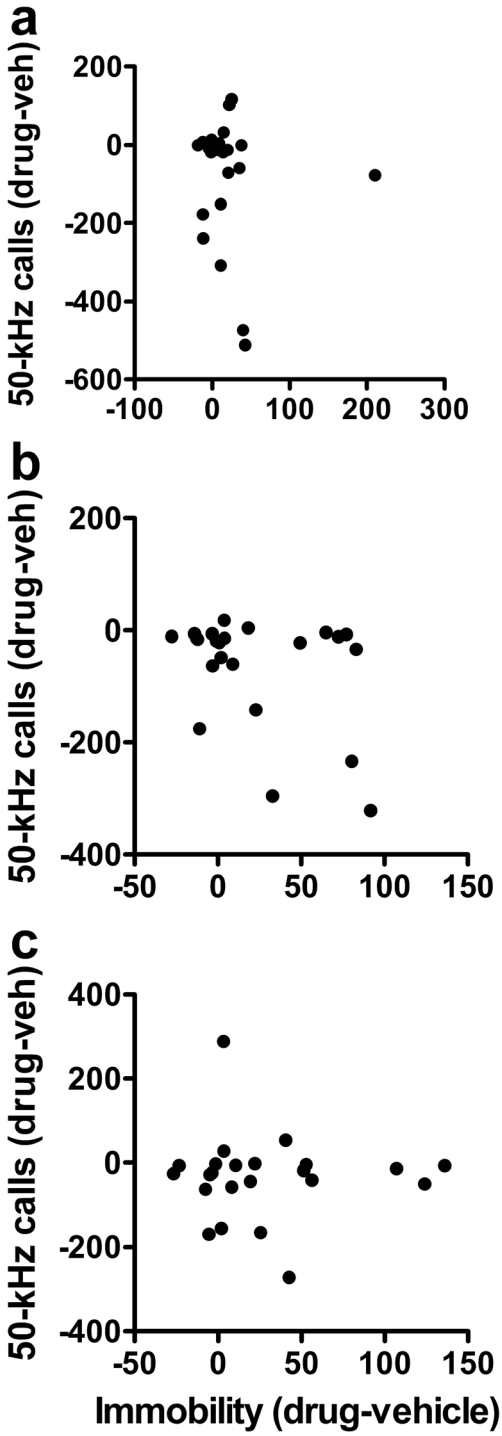

Fig. 4 Lack of relationship between drug effects on 50-kHz call rate and immobility duration, shown for FG 7142, PTZ, and mCPP (**a**–**c**, respectively). *Y*-axes show the number of calls per 10-min session, expressed as drug-minus-vehicle scores. *X*-axes show the vehicle-subtracted total time (s) per session spent immobile. Each point represents a single rat ($n = 23$)

Discussion

In the present study, we sought to determine whether drug-induced anxiety is accompanied by a characteristic shift in the 50-kHz call rate or subtype profile. Instead, the various anxiogenic drugs differed in terms of their effects on the 50-kHz call subtype prevalence as well as on the call rate. In addition, 22-kHz calls were notably sparse. Finally, no anxiogenic drug produced an amphetamine-like shift in the 50-kHz call profile.

Anxiogenic drug effects on 50-kHz vocalizations

The effects of anxiogenic drugs have received little attention in the context of 50-kHz vocalizations (see Introduction), with apparently no published reports concerning FG 7142, mCPP, or PTZ. All three drugs decreased the 50-kHz call rate. In contrast, caffeine markedly stimulated calling, confirming a previously reported trend (Simola et al. 2016; Simola et al. 2010), whereas yohimbine did not alter the 50-kHz call rate, also consistent with a previous report (Mahler et al. 2013).

The present study provides the first detailed analysis of individual 50-kHz call subtypes emitted by rats that are acutely challenged with anxiogenic drugs. The five anxiogenic test drugs exerted strikingly few effects on the relative prevalence of flat or trill call subtypes. Specifically, mCPP decreased the proportion of trill calls, whereas the proportion of flat calls was significantly decreased by yohimbine and caffeine (Table 1). Among anxiogenic drugs, it appears that only caffeine has previously been investigated with respect to 50-kHz call categories (Simola et al. 2010); in this earlier study, caffeine did not detectably change the relative prevalence of flat or trill calls, but these two categories were more broadly defined than in the present study.

Anxiety-like behavior vs. 50-kHz vocalizations

For each of the five anxiogenic drugs, we selected a dose and post-injection interval that has been reported to produce anxiety-like behavior in multiple behavioral assays (see Table 1 for examples, with corresponding literature citations in Supplementary Table 1). We measured immobility duration as an indicator of freezing-associated anxiety. Immobility was increased by three of the five test drugs (i.e., FG 7142, mCPP, and PTZ), but not by caffeine or yohimbine. Caffeine only marginally increased immobility or freezing at comparable doses in previous studies (Antoniou et al. 1998; Bhattacharya et al. 1997); quite possibly, immobility-related measures were contaminated by the locomotor stimulant effect of this drug. Yohimbine increased immobility in two previous reports (Bhattacharya et al. 1997; Park et al. 2001), but appears to perform inconsistently across behavioral assays, exerting anxiogenic-like effects in the elevated plus maze and social interaction test (e.g., Bhattacharya et al. 1997) but not in certain other test procedures (Baldwin et al. 1989; Jones et al. 2002; Molewijk et al. 1995).

In conclusion, only three of the five test drugs convincingly increased anxiety in the present study: FG 7142, mCPP, and PTZ. Of these, only mCPP significantly altered the prevalence of either flat or trill calls (Table 1), and no other call subtype appeared consistently affected. The only shared effect of these three drugs on 50-kHz call emission was a reduction in call rate. However, this shared inhibitory effect was not significantly correlated with drug-induced immobility and hence is unlikely to reflect anxiety.

Drug-induced aversion vs. 50-kHz vocalizations

At sufficiently high doses, most or all of the anxiogenic drugs that we tested produce not only anxiety but also extreme dysphoria in normal human subjects; such effects have been reported for FG 7142 (Dorow et al. 1983), mCPP (Charney et al. 1987; Murphy et al. 1989), PTZ (Good 1940; Rodin 1958), and yohimbine (Holmberg and Gershon 1961). However, strongly anxiogenic doses of caffeine and yohimbine do not always produce significant distress (Charney et al. 1983; Foltin and Fischman 1991; Lader 1969). Hence, the relationship between drug-induced anxiety and aversion is not always straightforward.

A similar complexity is seen in studies using adult rats, where anxiogenic drugs do not consistently produce conditioned place aversion (CPA), when given acutely at doses relevant to the present study. For example, neither FG 7142 nor mCPP produced a detectable CPA (Di Scala and Sandner 1989; Rocha et al. 1993), and mixed results were obtained with both PTZ (Bespalov 1996; Gauvin et al. 1991) and yohimbine (Chen et al. 2015; File 1986). Caffeine, in contrast, consistently produced a CPA (Brockwell et al. 1991; Patkina and Zvartau 1998; Steigerwald et al. 1988). The latter finding is potentially significant, since caffeine reduced the proportion of flat calls, an effect previously seen with *rewarding* drugs, i.e., amphetamine (Wright et al. 2010), cocaine (Wright et al. 2012), and morphine (Best et al. 2017). In future studies, therefore, it will be important to directly compare the effects of caffeine on 50-kHz call emission within the CPA procedure.

Amphetamine and 50-kHz vocalizations: relation to anxiogenic drugs

In the present study, acute amphetamine administration increased the 50-kHz call rate and increased the relative prevalence of 50-kHz trills, at the expense of flat calls, thus confirming previous studies (Wright et al. 2012; Wright et al. 2010). A shift in favor of the trill call subtype is also associated with cocaine and morphine administration, and has been proposed as an index of positive affect (Best et al. 2017; Wright et al. 2012, Wright et al. 2010). In the present study, the three most clearly anxiogenic drugs (i.e., FG 7142, mCPP, and PTZ) failed to exert amphetamine-like effects on any USV-related measure. This finding indicates that the effects of amphetamine on 50-kHz call emission do not reflect anxiogenic effects that are sometimes associated with this drug (Foltin and Fischman 1991); see Supplementary Table 1.

Drug effects on 22-kHz vocalizations

Adult rat 22-kHz "alarm/distress" calls have been widely used as a measure of anxiety or fear in adult rats, typically in conjunction with stressors such as air puffs, footshock, or acoustic startle (Sanchez 2003; Simola 2015). In this context, it has been proposed that 22-kHz calls reflect a "refractory, socially withdrawn or helpless state" (Sanchez 2003). In the present study, the anxiogenic drugs were tested in the absence of additional aversive stimuli, apart from the brief social isolation that occurred during the 10-min test session. Under these conditions, virtually, no 22-kHz calls were observed. These negative findings are consistent with previous studies using comparable doses of FG 7142 (Jelen et al. 2003), yohimbine (Mahler et al. 2013), PTZ (Portavella et al. 1993), and caffeine (Simola et al. 2010); mCPP does not appear to have been tested alone previously. While it remains possible that higher anxiogenic drug doses would have evoked 22-kHz vocalizations, as also found with higher-intensity electric footshock (Wöhr et al. 2005), the present findings nevertheless provide further evidence that anxiety per se is not generally sufficient to evoke 22-kHz vocalizations.

Study strengths and limitations

Study strengths include the detailed analysis of 50-kHz call subtypes, and the relatively powerful experimental design (i.e., 23 rats, repeated measures format). Limitations are as follows. First, testing rats individually was likely suboptimal, to the extent that ultrasonic vocalizations play a communicative role. However, while the effects of anxiogenic drugs would be worth reevaluating in a group setting, it is technically challenging to identify the specific animal emitting ultrasonic calls. Second, the open-field recordings yielded spectrograms that were somewhat less well-defined than those previously obtained in operant chambers (Wright et al. 2010), and hence acoustic properties such as bandwidth and frequency were not analyzed. Such an analysis would have been desirable, particularly since caffeine administration has been reported to affect these measures (Simola et al. 2010). Third, each drug was tested at only a single dose, and higher levels of anxiety might have produced a wider range of effects on USV emission. Nevertheless, all of our selected doses have been consistently reported to produce anxiety-like behavior in multiple studies, as documented in Supplemental Table 1, and all five anxiogenic drugs are associated with monotonic rather than inverted-U dose-response relationships (Bagdy et al.

2001; Baldwin and File 1989; Bhattacharya et al. 1997; Cole et al. 1995; File et al. 1988; File and Lister 1984; File et al. 1985; Wallis and Lal 1998). Fourth, each rat was exposed to several different drugs, and to repeated doses of amphetamine. While the experimental design was counterbalanced for carryover effects, this high degree of drug exposure may limit generalization to other studies; for example, with repeated drug administration, rats can become sensitized to amphetamine's acute effects on 50-kHz USV emission (Ahrens et al. 2009; Taracha et al. 2014). Lastly, our video tracking-based measure of anxiety (i.e., immobility duration) only approximates freezing behavior since the latter is commonly defined by a *crouched* immobile posture. However, the three test drugs which significantly increased immobility duration (FG 7142, mCPP, and PTZ) appear reliably anxiogenic at comparable doses.

Conclusions

In adult male rats, drug-induced anxiety is not sufficient to elicit 22-kHz calls but may be associated with reduced 50-kHz call emission when psychomotor stimulant effects are not present. Anxiogenic drugs, at moderate doses, do not selectively elicit any particular 50-kHz call subtype. Effects of amphetamine on 50-kHz call rate and subtype profile do not reflect anxiety.

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Compliance with ethical standards

All experiments comply with the current laws of Canada.

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