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ORIGINAL INVESTIGATION

Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context

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Abstract
Rationale 50-kHz ultrasonic vocalizations (USVs) emitted by adult rats are heterogeneous; they occur over a wide frequency range, show varying degrees of frequency modulation, and appear to differ in their behavioral significance. However, they have not been extensively categorized.
Objectives The main objective of this study was to identify subtypes of 50-kHz USVs emitted by adult rats and to determine how amphetamine (AMPH) or social testing condition affects their relative and absolute production rate and acoustic characteristics. A second objective was to determine the extent of individual differences in call rate, call subtype profile, and acoustic parameters (i.e., duration, bandwidth, and mean peak frequency).
Methods Adult male Long–Evans rats were administered systemic amphetamine (0.25–2 mg/kg, IP) and tested individually or with a cage mate for 20 min. Call categories were defined based on visual inspection of over 20,000 USV spectrograms. Surgical devocalization was performed on a subset of AMPH-tested rats in order to confirm the authenticity of call subtypes.
Results Fourteen categories of 50-kHz USVs were recognized. Call subtypes were differentially affected by social context, AMPH dose, and time within session. In contrast, the acoustic characteristics of call subtypes were notably stable. Marked and stable inter-individual differences occurred with respect to overall 50-kHz call rate, acoustic parameters, and call profile.
Conclusions The present findings, obtained under saline and amphetamine test conditions, provide the first detailed classification of adult rat 50-kHz USVs. Consideration of 50-kHz USV subtypes may advance our understanding of inter-rat communication and affective state.

Keywords Ultrasonic vocalizations · Amphetamine · Reward · Frequency-modulated · Trill · Dose–response · Individual differences

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Introduction

Ultrasonic vocalizations (USVs) have been observed in a number of rodent species (Sales 1972). In adult laboratory rats, two main types of USVs have been described: 22-kHz and 50-kHz calls (see Brudzynski 2009 for review). The 22-kHz call type has been termed a distress or "alarm" vocalization (Litvin et al. 2007), as it can be elicited by the presentation of a predator, painful stimuli, startling noises, and intermale aggression (Blanchard et al. 1991; Calvino et al. 1996; Han et al. 2005; Kaltwasser 1991; Thomas et al. 1983). In contrast, calls of the 50-kHz category have been detected in naturalistic appetitive contexts, such as during play, mating behavior,

exploratory activity, or in anticipation of food reward (Burgdorf et al. 2000; Knutson et al. 1998; Sales 1972). 50-kHz calls have also been elicited by several non-natural appetitive stimuli, particularly rewarding electrical brain stimulation and amphetamine (AMPH) administration (Ahrens et al. 2009; Burgdorf et al. 2000, 2001a, 2007; Simola et al. 2009; Thompson et al. 2006; Wintink and Brudzynski 2001). Of note, the 50-kHz class of calls encompasses a wide frequency range (30–90 kHz) (Kaltwasser 1990; Sales and Pye 1974), and these calls vary considerably in spectrographic structure (see below).

Until recently, rodent USVs were typically detected by means of frequency-division or heterodyne recording devices. These approaches allowed counting of calls but provided little or no information about acoustic parameters (Parsons 2000). In contrast, the use of high-frequency sampling of untransformed microphone signals has shown that adult rat 50-kHz USVs are heterogeneous, comprising "flat" (i.e., constant frequency) and "frequency-modulated" (FM) calls (Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006; Ciucci et al. 2009; Simola et al. 2009; Wohr et al. 2008). Somewhat more detailed classification schemes have been described, each comprising three or four subtypes of 50-kHz calls (Kaltwasser 1990; Vivian and Miczek 1993; White et al. 1990), but individual spectrograms seem to indicate a much richer diversity (Burgdorf et al. 2008a; Ciucci et al. 2009; Schwarting et al. 2007; Wohr et al. 2008). Recently, five USV subtypes were defined in adult mice (Panksepp et al. 2007) and as many as ten USV subtypes were documented in mouse pups (Scattoni et al. 2008), suggesting that a detailed classification of adult laboratory rat USVs would also be warranted.

Adult rat USVs can play a communicative role, as evidenced by the effects of devocalization and USV playback on rat behavior (Brudzynski and Chiu 1995; Burgdorf et al. 2008a; Thomas et al. 1981; Wohr and Schwarting 2007). Interestingly, FM and flat calls appear to differ in their neurochemical basis and behavioral significance (Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006). For example, FM calls have been suggested to signal a dopamine-dependent reward state (Burgdorf et al. 2008a) and, on preliminary evidence, are increased by the prototypical DA agonist AMPH more than flat calls (Simola et al. 2009). In contrast, flat calls may serve a social-coordinating function (Wohr et al. 2008). Rat 50-kHz USVs have frequently been detected even in the absence of conspecifics (e.g. Burgdorf et al. 2000; Schwarting et al. 2007), but whether rats make the same kinds of 50-kHz USVs when tested singly vs. paired with a conspecific has not, to our knowledge, been investigated.

Stable and pronounced inter-individual differences with respect to USV production rates have been noted in several studies (Burgdorf et al. 2001b; Mallo et al. 2007; Schwarting et al. 2007; Wohr et al. 2008, 2009; Wohr and Schwarting 2009). However, it has not been reported whether individual rats differ in terms of the specific call subtypes that they preferentially emit, that is, whether each rat possesses a characteristic "call profile". In addition, there have been no reports of stable differences in acoustic parameters of calls.

The present study therefore addressed the following hypotheses: (1) the rich heterogeneity of 50-kHz USVs is captured within a moderate number of discrete call categories, (2) call categories are differentially modulated by AMPH treatment and the presence of conspecifics, and (3) individual differences exist, not only in terms of the overall number of 50-kHz calls emitted, as previously shown, but also in relation to call subtypes and acoustic parameters. A final experiment was performed in order to confirm the authenticity of individual 50-kHz USV subtypes by means of a surgical devocalization procedure (Roberts 1975; White and Barfield 1990).

Methods

Subjects

In Experiment 1, subjects were 24 experimentally-naïve male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 319–380 g at the beginning of the experiment. For Experiment 2, subjects were 36 experimentally naïve male Long–Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 267–330 g at the start of the experiment. Subjects were housed three (Experiment 1) or two (Experiment 2) 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. The rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle. Food and water were available ad libitum, except during testing. Before the start of the experiment, animals were handled for approximately 3 min daily for 2 days. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Acoustic data acquisition, analysis, and classification of ultrasonic vocalizations

Testing took place in clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St. Albans, VT), each of which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). Electret microphones with a frequency response range of 15 to 125 kHz (FG-23329-C05, Knowles Acoustic, Itasca, IL) were securely placed through small holes

located centrally in the top panels of the experimental chambers. Consequently, the microphones were 15–30 cm from the rats during testing. Microphone signals were fed into a preamplifier (QuadMic, RME, Germany) and an anti-aliasing filter (Krohn-Hite 3323, Brockton, MA) before the analog signal was digitized through an A/D card (PCI-6251, National Instruments, Austin, TX) with a sampling rate of 200-kHz and a 16-bit resolution.

Acoustical analysis of the recordings was performed using Avisoft SASLab Pro (Version 4.2, Avisoft Bioacoustics, Berlin, Germany). Spectrograms were generated with a fast Fourier transform (FFT)-length of 512 points and an overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 390 Hz and a time resolution of 0.64 ms. Calls were selected manually with section labels and classified based on pre-defined frequency pattern criteria (see below); call classification was performed masked to the treatment condition. Three acoustic properties of each call were determined by the automatic parameter measurements feature of the software: duration, bandwidth (i.e., difference between the maximum and minimum peak frequency), and mean peak frequency (i.e., time-averaged peak energy frequency within each call). In order to improve accuracy of parameter measurements by the software, a threshold between −40 and −50 dB was set (setting: "Reject if peak amplitude <"). If background noise still interfered with proper measurement of acoustic parameters, those calls were discarded from the parameter analysis.

Each ultrasonic "call" had to meet three spectrographic criteria: (1) temporal continuity (i.e., maximal interruption of 20 ms), (2) fundamental frequency between 20 and 95 kHz, and (3) sound intensity and structure that was clearly distinct from background noise when seen under optimal viewing settings. From a visual inspection of over 20,000 USV spectrograms, 15 call categories were recognized (see Fig. 1):

(1) *Complex*: contain two or more directional changes in frequency of at least 3 kHz each
(2) *Upward ramp*: monotonically increasing in frequency, with a mean slope not less than 0.2 kHz/ms
(3) *Downward ramp*: monotonically decreasing in frequency, with a mean negative slope not less than 0.2 kHz/ms
(4) *Flat*: near-constant frequency greater than 30 kHz with a mean slope between −0.2 and 0.2 kHz/ms
(5) *Short*: duration less than 12 ms
(6) *Split*: middle component "jumps" to a lower frequency and contains a harmonic
(7) *Step up*: instantaneous frequency change to a higher frequency
(8) *Step down*: instantaneous frequency change to a lower frequency
(9) *Multi-step*: two or more instantaneous frequency changes
(10) *Trill*: rapid frequency oscillations with a period of approximately 15 ms (either sinusoidal or appearing as repeated "inverted-Us").
(11) *Flat/trill combination*: a trill that is flanked on one or both sides by a monotonic portion that is no less than 10 ms
(12) *Trill with jumps*: a trill that contains one or more higher-frequency components
(13) *Inverted U*: a monotonic increase followed by a monotonic frequency decrease, each of at least 5 kHz
(14) *Composite*: calls (other than flat/trill combinations) that comprise two or more categories
(15) *22-kHz calls*: near-constant frequency calls between 20 and 25 kHz

Finally, a few (1%) other calls were classified as "miscellaneous" because they did not fit any of the above call categories. A proportion of calls (7%) was spectrographically unclear and categorized as "unclassifiable".

Inter-rater reliability in call category identification was assessed using a subset of 500 calls, which was independently rated by the main experimenter (J.M.W.) and a trained student. Reliability was high (Cohen's kappa=0.95), such that 96% of calls received the same classification from both individuals. Intra-rater reliability, assessed by a repeat scoring of 500 calls by the main experimenter one week apart, was also high (Cohen's kappa=0.96), such that 97% of calls were assigned the same classification between scorings.

Drugs

D-Amphetamine sulfate (Sigma Aldrich, Oakville, ON) was dissolved in sterile 0.9% saline and administered by IP injection in a volume of 1 ml/kg; doses are expressed as salt.

Experimental protocol

Experiment 1: Systemic AMPH dose–response in singly- and pair-tested rats

The experiment comprised an initial habituation day followed immediately by six test days, spaced 2 days apart in order to minimize possible carry-over effects of the drug. Rats were housed three per cage: one rat from each home cage (n=8 rats) was randomly assigned to be tested singly throughout the experiment, and the other two rats from the same home cage were always tested together (n=8 pairs). On the first day (Habituation), rats were placed in the test chambers for 10 min. Over the six test days, each rat or rat

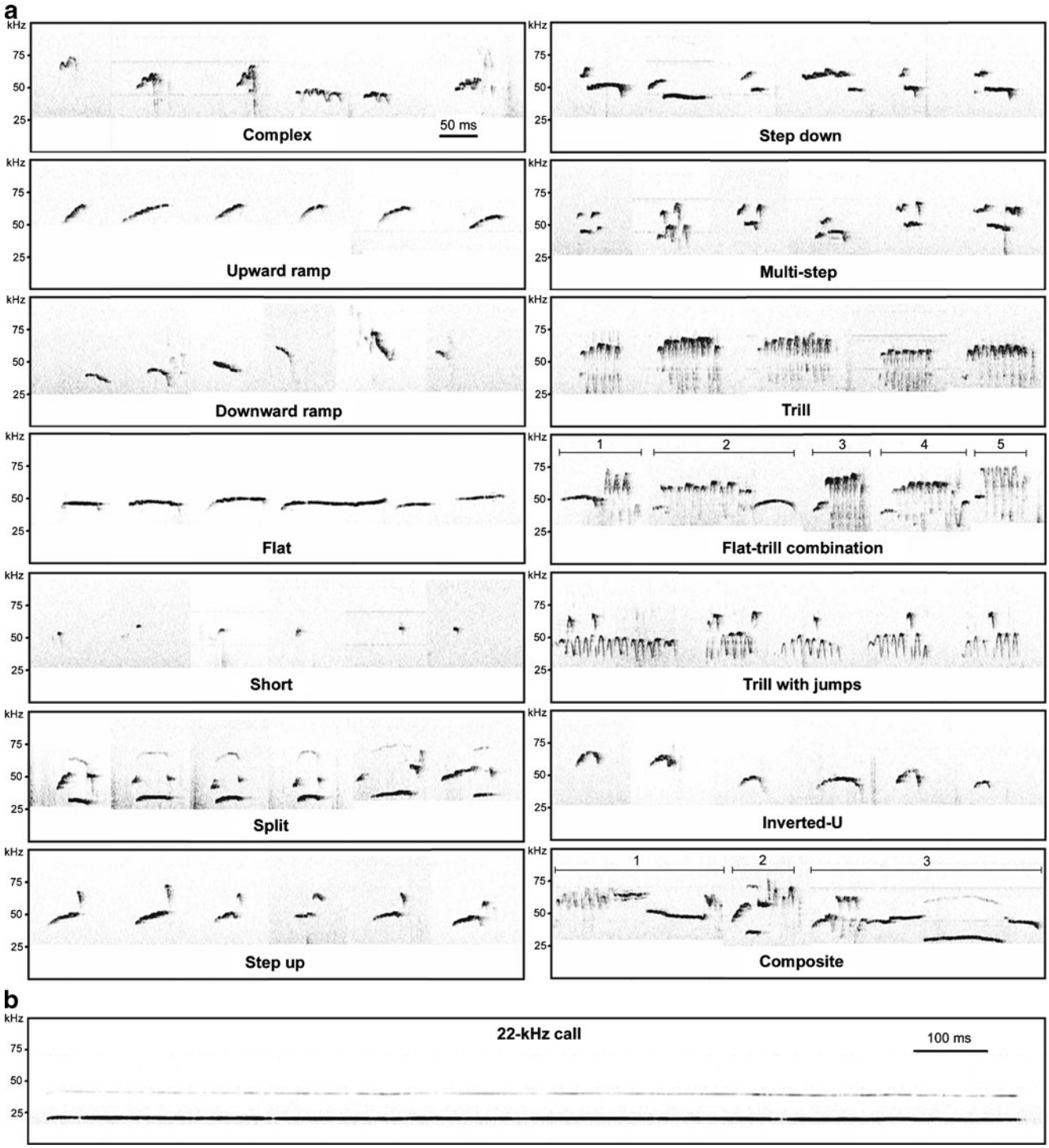

Fig. 1 Representative calls for each of the 14 categories of 50-kHz USVs (**a**) and a 22-kHz USV (**b**). Several exemplar calls are shown for each 50-kHz call category; these examples are not necessarily consecutive nor made by the same rat. Individual "flat–trill combination" and "composite" calls are differentiated by the accompanying line brackets. The time scale for all 50-kHz calls is indicated in the top left panel

pair received two administrations of saline and all four doses of amphetamine (0.25, 0.5, 1, and 2 mg/kg), counterbalanced as fully as possible. Immediately following drug administration, the animals were placed in the test chambers and recorded for 20 min.

Experiment 2: Effects of surgical devocalization

This experiment was conducted in two parts (using 16 and 20 subjects, respectively). Rats were initially tested with AMPH in order to screen out low-rate callers as follows. Every 2 days

over 5 days, each rat (n=36) received an IP injection of AMPH (1 mg/kg) and was immediately placed in the test chamber alone and recorded for 20 min. The 14 rats with the lowest number of USVs were excluded from the study. The remaining 22 rats were randomly allocated to two groups of comparable pre-surgery call rates (mean±SEM 73±7 vs. 77±6 calls/min). One group received devocalization surgery; the other was sham-operated (see "Surgery"). Following a 6-day recovery period, rats received one additional test session under AMPH. One sham-operated rat was excluded from analysis since data collection failed in the post-surgery session due to a technical problem.

Surgery

Under general anesthesia, achieved by 2–4% isoflurane in conjunction with pure oxygen, a 2-cm incision was made on the ventral surface of the neck. Local anesthesia was provided by infiltration of lidocaine. The sternohyoideus muscle was separated to expose the trachea and locate the recurrent laryngeal nerves. Once located, approximately 3 mm of the nerve on both sides (bilateral section) was removed (devocalized), or left intact (sham). The incision was closed with subcutaneous suture and 1–2 staples. Analgesic carprofen (5 mg/kg SC) was administered to rats at surgery and again every 24 h for the following 3 days. One devocalized rat died the day following surgery. The rats were allowed to recover for 6 days before testing.

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS Inc., Chicago, Illinois). The number of USVs acquired for the pair-tested rats was divided by two in order to express the results on a per-rat basis. Only USVs that occurred during minutes 3, 8, 13, and 18 (Experiment 1) or during minutes 13 and 18 (Experiment 2) of each 20-min recording were selected. When specifically examining the effect of AMPH on USVs in Experiment 1, only the latter two sampled time intervals were used in order to allow time for AMPH to take effect. In this study, 22-kHz calls accounted for less than 1% of USVs and were not analyzed further.

The statistical analysis of acoustic parameters (duration, bandwidth, and mean peak frequency) was performed by repeated-measures analysis of variance (ANOVA). The fact that not all subjects emitted every call subtype under every condition posed a challenge, since this type of ANOVA discards subjects with *any* missing values. As a first step, we excluded from analysis four call categories as well as four singly-tested rats, which had a large number of missing values. Subsequently, the missing values for each remaining rat were replaced by the mean of its other test conditions. In total, 5% of the raw data values were interpolated.

Data from the two saline test sessions were analyzed as follows. For the correlation of the total number of calls between the two saline tests, Spearman's correlation coefficient was calculated, and unprotected paired t tests were performed for each call category. For the remainder of the analysis, the number of calls was averaged across the first and second saline test. ANOVA was performed to test the effects of "group" (i.e., between subjects; singly- *vs.* pair-tested), call "category" (within subjects; 14 call subtypes), "time" (within subjects; minutes 3, 8, 13, and 18), "dose" (within subjects; five dose levels), and "rat" (between subjects; eight singly-tested and eight pair-tested rats), where appropriate, and was performed on each call subtype either in absolute terms, or as a proportion of all calls. Only call categories with a proportion greater than 2% in at least one of the groups were included in this analysis. Two-sample independent t tests or paired t tests were used where appropriate. All ANOVA p values were subject to the Huynh–Feldt correction where applicable. For all tests, a two-tailed p value less than 5% was considered significant.

Results

Experiment 1: Systemic AMPH dose–response in singly- and pair-tested rats

Classification of rat ultrasonic vocalization subtypes

Over 20,000 calls were examined in this experiment, from which 14 categories of 50-kHz calls were recognized; spectrograms of typical calls of each category are shown in Fig. 1. Three call subtypes were particularly prevalent, together comprising approximately 50% of the observed calls pooled across all experimental conditions: the trill (29% of calls), flat/trill combination (16%), and flat (14%). The least common 50-kHz subtype was the split call, accounting for only 0.5% of calls. Of the 93% of calls that were considered "classifiable" (see "Methods"), only 1% did not fall into one of the 14 categories. These infrequent miscellaneous calls varied widely in appearance.

Ultrasonic vocalizations during saline test sessions

All 50-kHz call categories occurred during saline test sessions. The total number of calls made by each rat during the first and second saline test were significantly correlated (Pearson r=0.63, df 14, p<0.01; Fig. 2a). The mean number of each call subtype did not differ significantly between the two saline tests in either the singly- or pair-tested rats. For all further analysis, the number of USVs was averaged across the two saline sessions.

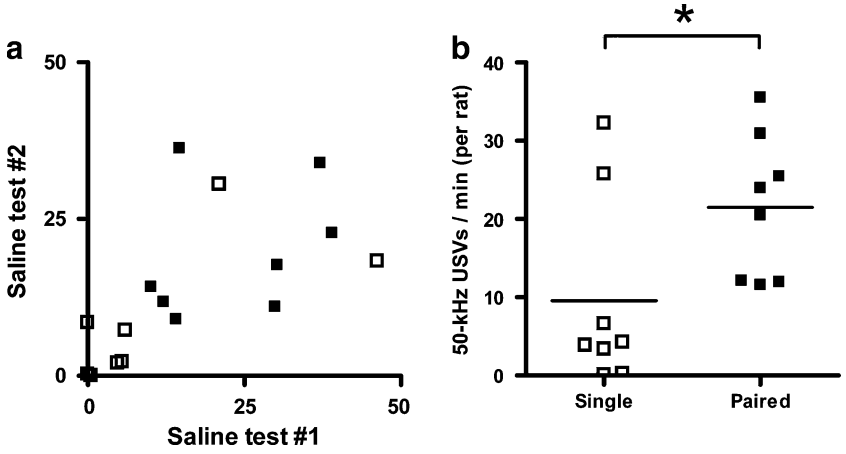

Fig. 2 Rate of 50-kHz calling in the two saline test sessions. Each rat or rat pair is represented by *open* or *closed squares*, respectively. The rate of 50-kHz calling was averaged across four time intervals (minutes 3, 8, 13, and 18 post-injection) and expressed as calls/min per rat. The 50-kHz call rate was significantly correlated between the first and second saline test sessions (Spearman $r=0.81$, df 16, $p<0.01$) (**a**). Pair-tested rats emitted significantly more 50-kHz USVs (per rat) than singly-tested rats during saline test sessions (**b**). *$p<0.05$, *t* test

During saline tests, pair-tested rats emitted approximately twice as many calls as singly-tested rats, on a per-rat basis (main effect of group: $F(1,14)=4.84$, $p<0.05$; Fig. 2b). The call profile differed markedly between singly- and pair-tested rats (Fig. 3). In particular, pair-tested rats emitted a significantly higher proportion of trills ($t=2.86$, df 10, $p<0.05$) and flat–trill combinations ($t=2.24$, df 14, $p<0.05$) than singly-tested rats. The proportion of flat calls appeared greater in the singly-tested rats, but this difference was not significant, perhaps because this measure was highly variable across singly-tested rats.

Effect of AMPH on ultrasonic vocalizations

AMPH dose-dependently increased the total number of 50-kHz USVs per minute ($F(4,56)=13.30$, $p<0.0001$; Fig. 4). Of note, all doses of AMPH tested, including 0.25 mg/kg, caused a significant increase in calls relative to the saline

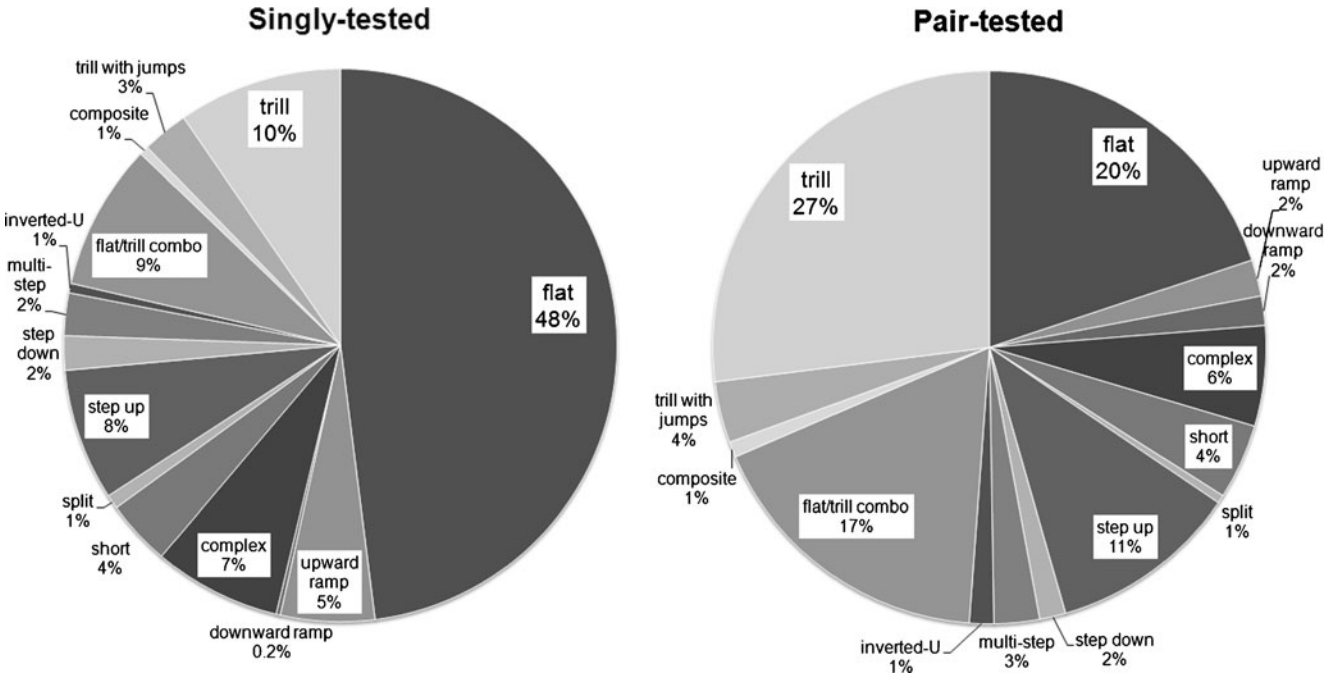

Fig. 3 Call profiles differed between singly-tested and pair-tested rats during saline test sessions. Each sector represents the group mean number of calls in a given category, expressed as a percentage of all classifiable calls ($n=8$ rats or rat pairs). Pair-tested rats emitted a significantly higher proportion of trills and flat–trill combinations than singly-tested rats (see text), whereas singly-tested rats tended to emit a higher percentage of flat calls

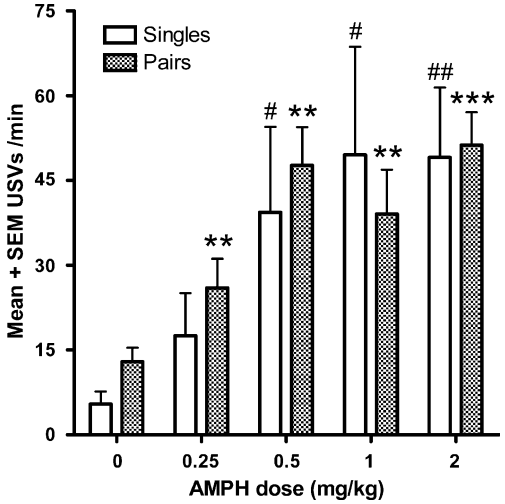

Fig. 4 AMPH dose-dependently increased the number of USVs emitted by singly- and pair-tested rats. The rate of 50-kHz calling was averaged across two time intervals (minutes 13 and 18 post-injection) and expressed as calls/min per rat. #$p<0.05$, ##$p<0.01$, **$p<0.01$, ***$p<0.001$ vs. corresponding saline condition (paired t tests; n= 8 rats or 8 rat pairs, respectively)

condition (paired t tests with Bonferroni correction, n=16, $p<0.01$–0.0001). The two groups evinced comparable absolute increases in call rate (per rat) in response to AMPH. In order to assess whether repeated AMPH administration induced behavioral sensitization, we examined whether the overall rate of calling increased across test days; however, no significant change was observed.

AMPH administration did not result in the emergence of new call types, but it clearly altered the call profile (Fig. 5; see Table 1 for statistical analyses). The relative proportion of trill calls dose-dependently increased with AMPH in both singly- and pair-tested rats. The other call types that were preferentially affected by AMPH were the flats and shorts. Similar to the saline condition, pair-tested rats emitted a significantly higher proportion of trills and trills with jumps than singly-tested rats, irrespective of the AMPH dose.

Effect of time within session

The overall call rate decreased over the test session ($F(3,42)$= 3.320, $p<0.05$), independently of AMPH dose or social condition (Fig. S1a). However, AMPH-induced calling (i.e., calls emitted under AMPH minus those under saline) was most pronounced during the last two sampled time intervals of the 20-min test session (i.e., minutes 13 and 18, Fig. S1b). The call profile also changed over time (category×time interaction, $F(39,507)=2.63$, $p<0.05$) irrespective of whether rats were tested singly or in pairs; the only significant time-dependent change was in the proportion of flat calls ($F(3,42)=5.31$, $p=0.02$), which became less frequent relative to other calls as the session progressed (i.e., minutes 3 vs. 8, 13, or 18: paired t tests, $p<0.05$).

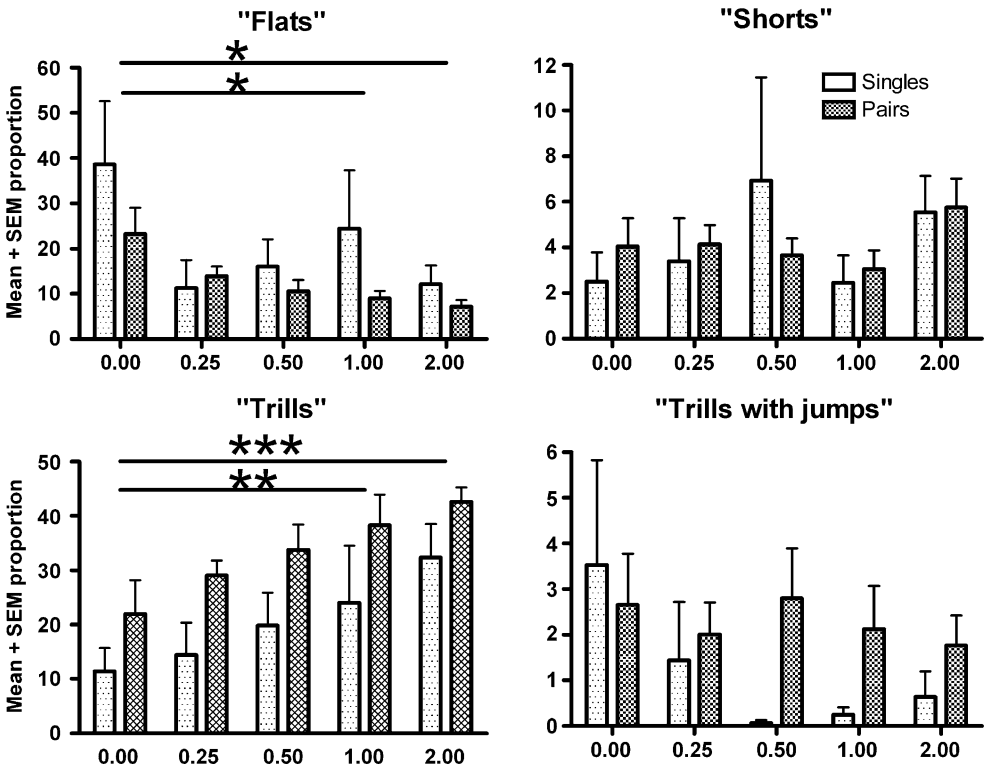

Fig. 5 Amphetamine dose-dependently increased the proportion of trill calls and suppressed flat calls. The proportion (mean±SEM) of each call category for singly- and pair-tested rats is plotted against increasing AMPH dose (only subtypes that exhibited significant changes under AMPH are shown; see supplemental Fig. S4 for *all* call categories). Note also the overall higher proportion of trills and trills with jumps in the pair-tested rats compared to the singly-tested rats. Amphetamine-induced changes with respect to flat, short, and trill calls were not significantly group-dependent (Table 1); pooling the two groups (i.e., single and pair-tested), significant differences from the saline condition are indicated by asterisks (*$p<0.05$, **$p<0.01$, ***$p<0.001$, paired t tests,

Table 1 ANOVA results for the call profile data (i.e. proportion of calls in each category)

Call type	S/P F (1,12)	AMPH F (4,48)	S/P×AMPH F (4,48)
Complex	1.75	0.93	0.37
Upward ramp	1.44	0.21	0.10
Downward ramp	0.00	0.69	1.17
Flat	1.34	**4.05***	0.29
Short	1.07	**3.15***	0.74
Split	n/a	n/a	n/a
Step up	0.12	1.34	**2.94***
Step down	1.17	1.33	1.34
Multi-step	1.94	1.56	**4.18****
Trill	**6.26***	**4.63****	0.21
Flat/trill combo	0.32	0.77	0.92
Trill with jumps	**4.91***	0.96	0.40
Inverted U	3.77	1.25	0.95
Composite	0.99	1.31	1.08

Two-way analyses of variance (ANOVAs) were performed separately for each call subtype, with between-subjects factor S/P (singly- vs. pair-tested), and within-subjects factor AMPH (i.e., dose of amphetamine). n/a, split calls were excluded from this analysis (see "Methods"). n=8 (rats or rat pairs). Significant F values are shown in bold *p< 0.05, **p< 0.01. Corresponding data are shown in Fig. 5

Correlation between call subtypes

To test whether rats that preferentially emitted a particular call subtype would also preferentially emit (or avoid emitting) any other call subtypes, we performed an exploratory correlational analysis (Table S1). Among the more frequent call subtypes, there was a significant positive correlation between step-ups and flats and between step-ups and flat–trill combinations. In contrast, a significant negative correlation occurred between trills and flats and between trills and step-ups.

Acoustic parameters (duration, bandwidth, and mean peak frequency)

Acoustic data and related statistical analyses are presented in Tables 2 and S2. Acoustic parameters were largely unaffected by social testing condition and AMPH dose (Table S2). The only exception was a slight (approximately 2 kHz) but significant decrease in bandwidth at the lowest dose of AMPH relative to saline (not shown). There was no difference, in any acoustic parameter examined, between singly-tested and pair-tested rats. Not surprisingly, acoustic parameters differed markedly between 50-kHz call subtypes (main effects of call category: p< 0.0001). All 50-kHz USV subtypes had a mean peak frequency between 48 and

Table 2 Call parameters (duration, bandwidth, and mean peak frequency) of each call category

Category	Duration (ms)				Bandwidth (kHz)				Mean peak frequency (kHz)			
	n	Mean	SEM	5th and 95th percentile	n	Mean	SEM	5th and 95th percentile	n	Mean	SEM	5th and 95th percentile
Complex	1,017	28.0	0.32	15.3, 47.3	972	9.1	0.2	3.5, 25.0	996	54.6	0.2	41.1, 64.9
Upward ramp	395	30.3	0.57	15.3, 52.3	378	7.9	0.3	3.5, 19.5	383	52.1	0.3	42.5, 61.8
Downward ramp	88	23.9	1.41	10.2, 55.1	77	8.7	0.8	3.1, 25.5	83	50.4	1.0	37.2, 65.1
Flat	2,595	34.8	0.44	13.4, 70.2	2,401	3.5	0.0	0.4, 7.5	2,460	48.8	0.1	40.2, 59.3
Short	657	9.6	0.07	7.0, 12.1	575	1.7	0.1	0.4, 4.2	624	57.3	0.3	42.2, 67.9
Split	51	105.5	19.26	37.9, 388.4	51	26.5	1.2	14.5, 39.8	51	40.3	0.9	31.2, 51.4
Step up	1,649	34.7	0.30	16.6, 55.6	1592	19.0	0.1	12.1, 27.8	1,617	52.9	0.1	44.2, 62.9
Step down	222	36.6	1.30	16.0, 71.2	219	16.3	0.5	7.8, 31.9	222	52.0	0.4	41.6, 62.2
Multi-step	439	39.1	0.66	19.2, 66.9	434	19.2	0.3	11.9, 33.1	437	53.4	0.3	41.5, 64.5
Trill	4,773	45.7	0.31	20.4, 83.8	4528	15.0	0.2	3.5, 40.2	4,644	59.9	0.1	48.2, 68.7
Flat–trill	2,923	60.6	0.51	30.0, 114.5	2874	22.2	0.2	12.8, 39.1	2,902	55.1	0.1	45.7, 64.0
Trill with jumps	383	67.5	1.62	31.3, 129.5	378	23.9	0.5	13.3, 43.7	382	54.4	0.3	42.6, 64.4
Inverted U	106	27.1	1.04	15.2, 45.9	99	9.7	0.8	5.1, 29.3	103	51.7	0.7	42.1, 64.6
Composite	191	87.2	3.60	36.4, 192.4	191	25.5	0.8	12.5, 46.1	191	55.3	0.5	42.7, 64.9
22-kHz	117	795.2	49.54	218, 1,910	117	9.4	0.7	1.5, 22.3	117	21.9	0.2	18.7, 25.1

Calls are from all testing conditions in Experiment 1. If background noise interfered with accurate measurement of acoustic parameters by the software, those calls were excluded from analysis (see "Methods"). In some cases, determination of only a subset of parameters was possible for a given call

60 kHz, except split calls, whose mean peak frequency was considerably lower (mean=40.3 kHz, see Table 2). Split calls were also longer than other 50-kHz USV subtypes, with a mean duration of 105 ms vs. 10–90 ms for other call subtypes (Table 2).

Individual differences in rate of calling, acoustic parameters, and call profile

Pronounced individual differences occurred in all three respects. First, individual rats differed consistently in terms of rate of calling. This was the case both within sessions and across AMPH doses (Cronbach's alpha=0.979 and 0.907, respectively; Fig. S2a, b). Analogous differences occurred between rat pairs (Cronbach's alpha=0.862 and 0.844, respectively; Fig. S2c, d). Second, individual differences in *acoustic parameters* (duration, bandwidth, and mean peak frequency) were found for almost every 50-kHz USV subtype; this is illustrated for trills and flats in Fig. 6. Finally, individual rats differed in their *call profiles* (rat× category interaction, $F(91,377)=1.68$, $p<0.05$; Fig. S3), and analogous differences were found between dyads of pair-tested rats ($F(91,416)=2.08$, $p<0.01$).

Experiment 2: Devocalization

Rats that were ultimately selected for surgery emitted 43–113 USVs/min during the pre-surgery AMPH screen. A comparison of the pre- and post-surgery AMPH tests showed that devocalization surgery suppressed USVs (group×time interaction: $F(1,18)=25.87$, $p<0.001$); the devocalized group emitted significantly fewer calls (paired $t=6.25$, df 3, $p<0.0001$) following surgery, whereas the rate of USVs emitted by sham-operated rats did not change significantly. Every call subtype was emitted by each group prior to surgery. USVs were completely abolished in 6 out of 10 devocalized rats, and the remaining rats emitted very few calls (mean±SEM 4±3 USVs/min) when tested under AMPH. Almost every 50-kHz call subtype was significantly reduced following devocalization surgery (Mann–Whitney *U* test, $p<0.05$–0.001). The only exceptions were the highly infrequent split and downward ramp calls; here, statistical power was limited. Sham surgery did not appear to alter the spectrographic appearance of 50-kHz calls or the call profile (data not shown); the low number of calls emitted by devocalized rats precluded analysis of these parameters. Finally, devocalized rats tended to gain less weight after

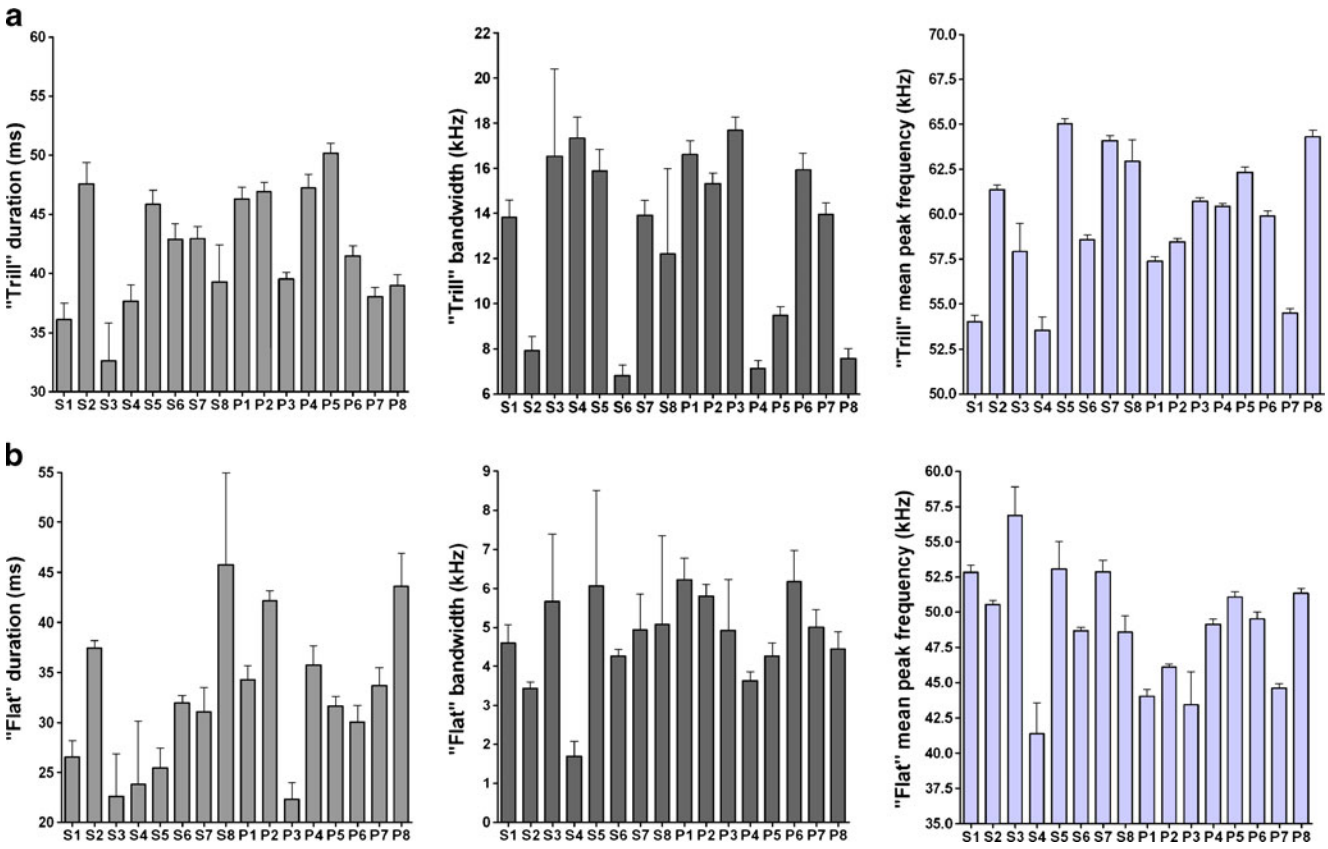

Fig. 6 Individual differences in acoustic parameters of 50-kHz calls. Differences in duration, bandwidth, and mean peak frequency were observed for every call subtype, and are exemplified here for trills (**a**) and flat calls (**b**). The *y*-axis variables are expressed as mean+SEM. The *x*-axis shows each singly-tested rat (S1–S8) and each pair-tested rat (P1–P8). One-way ANOVAs; $n=9$–760 calls; $p<0.0001$

surgery than the sham-operated rats (mean±SEM, respectively: 30.3±17.2 vs. 76.8±3.5 g; t=2.65, df 3, p=0.07).

Discussion

The present study provides the first detailed classification of adult rat 50-kHz USVs under saline and amphetamine test conditions. The authenticity of these calls was confirmed through surgical devocalization. Our analysis yielded several novel findings. Systemic amphetamine administration increased 50-kHz calling dose-dependently. More interestingly, call profiles (i.e., relative frequency of each call category) were differentially affected by social context, drug, and time within session. In particular: (1) pair-tested rats produced a higher proportion of trill calls than did singly-tested rats under both drug and saline conditions, (2) amphetamine altered the call profile such that trills became more prominent while flat calls became less so, and (3) flat calls became proportionately less frequent late in the session. In contrast, the acoustic characteristics of individual call subtypes (i.e., duration, bandwidth and mean peak frequency) were notably stable. Stable inter-individual differences were also found, not only with respect to overall 50 kHz call rate, as previously reported, but also in terms of acoustic parameters and call profile.

All the 14 USV subtypes described here are likely to be authentic rat vocalizations, since they virtually all disappeared after surgical transection of the recurrent laryngeal nerve. The origin of adult rat 50-kHz ultrasounds has been the subject of some debate, with suggestions that some calls might represent a byproduct of locomotion (Blumberg 1992) or serve a thermoregulatory role (Blumberg and Moltz 1987). The locomotor artifact hypothesis, however, has been countered by several lines of evidence (Knutson et al. 2002; Simola et al. 2009). For example, doses of caffeine that would be expected to increase locomotor activity are reported not to increase the rate of 50-kHz calling in adult rats (Simola et al. 2009). A thermoregulatory role of 22-kHz USVs has been proposed in adult rats, in the context of sexual behavior (Blumberg and Moltz 1987). As in the case of copulation, the higher doses of amphetamine used in the present study likely produced mild (1–2°C) hyperthermia in the periphery (Lin et al. 1980; Ulus et al. 1975) and therefore quite possibly in the brain as well. However, it seems unlikely that the increased rate of 50-kHz calling seen after amphetamine administration would reflect an attempt at thermoregulation, insomuch as these calls are much briefer and less intense than 22-kHz USVs.

Previous 50-kHz classification schemes have comprised only three or four subtypes (Kaltwasser 1990; Vivian and Miczek 1993; White et al. 1990). We now describe a considerably larger repertoire of calls. We cannot of course exclude the possible occurrence of additional call subtypes in other behavioral contexts, stages of development, or indeed by other rat strains. Importantly, our 14 call categories have nearly all been depicted previously (see Table 3), and they appear to capture all published spectrographic examples of rat 50-kHz USVs. Interestingly, eight of the ten USV subtypes that were recently described in mice appear to have acoustic counterparts in adult rats (compare Fig. 1 with Fig. 2 of Scattoni et al. 2008). This high degree of similarity possibly reflects physical constraints on call production; it remains to be determined whether these seemingly homologous call subtypes signal the same information in rats as in mice.

Automated call categorization has been achieved for a limited number of mouse USV subtypes (Holy and Guo 2005); a similar procedure for categorizing our 14 call subtypes would be highly desirable, for several reasons. First, manual categorization is highly labor-intensive. Second, although categorization was usually unambiguous (as reflected in high inter- and intra-rater reliabilities), mathematical modeling of 50-kHz calls could potentially eliminate any observer bias. Third, a modeling procedure might reveal further heterogeneity within our existing classification scheme.

In the present study, paired cage mates called significantly more, on a per-rat basis, than rats tested alone under drug-free conditions. This result is in line with previous findings by Brudzynski and Pniak (2002) using unfamiliar conspecifics, and may be unsurprising given that USVs appear to have a communicative role (see "Introduction"). It should however be noted that our testing protocol did not distinguish between vocalizations emitted by individual pair-tested rats, and consequently, it is not clear whether each pair-tested rat was similarly affected by AMPH or cage-mate presence.

In our study, AMPH dose-dependently increased the rate of 50-kHz calling, consistent with previous investigations that employed single systemic AMPH doses (Ahrens et al. 2009; Simola et al. 2009; Wintink and Brudzynski 2001). Of note, even the lowest dose of AMPH tested (0.25 mg/kg), a dose considered to be a "low" behaviorally effective dose in rats (Grilly and Loveland 2001), caused a highly significant increase in USVs, indicating that USVs are a relatively sensitive behavioral measure.

While systemic administration of AMPH has been widely reported to increase 50-kHz calling, effects on 50-kHz *call subtypes* have received less attention. In particular, Ahrens et al. (2009) showed that trill, but not flat, calls increased with repeated AMPH administration. In another study, a single, relatively high dose of AMPH (2 mg/kg) increased the FM-flat ratio (Simola et al. 2009). By examining the effect of AMPH on each of the 14 subtypes of 50-kHz USVs, we found that AMPH dose-dependently increased the proportion of trill calls and decreased the

Table 3 The 50-kHz call categories defined in the present study (i.e., call subtype) in relation to published spectrographic evidence from adult rats

Call subtype	Sex	Rat strain	Age/weight	Condition
Complex	Male[a,b,c]	Sprague-Dawley,[a] Wistar[b,c]	192–226 g,[b] 220–260 g,[a] 280–310 g[c]	Intra-NAc carbachol,[a] heterospecific play,[b] cage exploration[c]
Upward ramp	Male[a,d,e]	Sprague–Dawley,[a] Wistar,[d] Long–Evans[e]	220–260 g,[a] 250–450 g,[d] 300–400 g[e]	Intra-NAc carbachol,[a] intracerebral glutamate,[d] aggression[e]
Downward ramp	Male[e]	Long–Evans[e]	300–400 g[e]	Aggression[e]
Flat[f,g]	Male[h,i,a,d,b,j,e,c,k]	Long-Evans,[h,i,j,e,l] Sprague–Dawley,[a] Wistar[d,b,c,k]	380–540 g,[a], 9–15 months,[i] 220–260 g,[a] 250–450 g,[d] 192–226 g,[b] 6–8 months,[j] 300–400 g,[e] 280–310 g[c,k]	IV AMPH,[h] sexual behavior,[i,l] intra-NAc carbachol,[a] intracerebral glutamate,[d] IP caffeine or AMPH,[j] heterospecific play,[b] aggression,[e] cage exploration[c,k]
Short				
Split	Male[i,a]	Long–Evans,[i] Sprague–Dawley[a]	9–15 months,[i] 220–260 g,[a]	Sexual behavior,[i] intra-NAc carbachol,[a]
Step up	Male[a]	Sprague–Dawley[a]	220–260 g[a]	Intra-NAc carbachol[a]
Step down	Male[a,c]	Sprague–Dawley,[a] Wistar[c]	220–260 g,[a] 280–310 g[c]	Intra-NAc carbachol,[a] cage exploration[c]
Multi-step	Male[a,m,k]	Black Rat,[m] Sprague–Dawley,[a] Wistar[k]	220–260 g,[a] 280–310 g[k]	Aggression,[m] intra-NAc carbachol,[a] cage exploration[k]
Trill	Male[b,j,k]	Black Rat,[m] Long–Evans,[j] Wistar[b,k]	192–226 g,[b] 6–8 months,[j] 280–310 g[k]	IP caffeine or AMPH,[j] cage exploration,[k] heterospecific play,[b] sexual behavior[m]
Flat–trill combination	Male[b]	Long–Evans,[l] Wistar[b]	192–226 g,[b]	Sexual behavior,[l] heterospecific play[b]
Trill with jumps	Male[h,n,i]	Long–Evans[h,n,i]	380–540 g,[h] 6 months,[n] 9–15 months[i]	IV AMPH,[h] sexual behavior[n,i]
Inverted U	Male[a]	Sprague–Dawley[a]	220–260 g[a]	Intra-NAc carbachol[a]
Composite[f,g]	Male[c,k]	Wistar[c,k]	280–310 g[c,k]	Cage exploration[c,k]

[a] Fendt et al. (2006); [b] Schwarting et al. (2007); [c] Wohr and Schwarting (2007); [d] Fu and Brudzynski (1994); [e] Vivian and Miczek (1993); [f] Burgdorf et al. (2007); [g] Burgdorf et al. (2008a, b); [h] Ahrens et al. (2009); [i] Ciucci et al. (2009); [j] Simola et al. (2009); [k] Wohr et al. (2008); [l] White et al. (1990); [m] Kaltwasser (1990); [n] Ciucci et al. (2007)

proportion of flat calls. Interestingly, the proportion of the other 12 subtypes of 50-kHz USVs remained stable across AMPH doses, although most 50-kHz subtypes significantly increased in absolute number.

Analysis of rodent 50-kHz USVs has tended to emphasize the calling rate rather than the spectrographic characteristics of calls; however, acoustic call parameters are also susceptible to experimental manipulation, in some cases independently of call rate (Ciucci et al. 2007; Hodgson et al. 2008; Simola et al. 2009; Vivian and Miczek 1993). It has been reported that neither AMPH administration (Simola et al. 2009; Thompson et al. 2006) nor social testing conditions (Brudzynski and Pniak 2002) alter the acoustic parameters of 50-kHz calls; however, in these studies, little (or no) distinction was made between call subtypes. We now show a similar lack of effect across multiple 50-kHz call categories, the only exception being a modest effect of AMPH on bandwidth.

Inter-rat differences in 50-kHz call rates have been widely reported in response to various stimuli (Burgdorf et al. 2001b, 2005, 2008b; Mallo et al. 2007; Schwarting et al. 2007; Wohr et al. 2008, 2009; Wohr and Schwarting 2009). In the present study, such differences were maintained not only within session but also across different AMPH doses. To further elucidate the relationship between 50-kHz calls and AMPH's rewarding effects, it would be of interest to determine whether individual differences in AMPH-induced calling rate predicts intravenous self-administration or the magnitude of conditioned place preference.

To our knowledge, this study is the first to report individual differences in call profiles (i.e., the proportion of calls in each category). Thus, in addition to differences in absolute call rate, some rats, for example, favored complex calls over trills (or vice versa). Furthermore, individual differences in acoustic parameters, previously reported for 22-kHz calls (van der Poel and Miczek 1991), were evident with respect to 50-kHz calls. Specifically, we found inter-rat differences in the duration, bandwidth, and mean peak frequency of each 50-kHz call subtype. Consequently, we speculate that the combination of call profile and acoustic parameters may be sufficient to allow rats to recognize individual conspecifics, even in the absence of odor cues.

Recent evidence suggests that 50-kHz call subtypes may differ in their behavioral significance; for example, FM calls, but not flat, have been associated with appetitive stimuli (e.g. Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006; Wohr et al. 2008). In the present study, we used two stimuli: AMPH and the opportunity to interact with another similar-aged rat, both of which have been shown to be rewarding (Calcagnetti and Schechter 1992; Spyraki et al. 1982). Only trill calls were proportionally increased by both these conditions (flat–trill combinations increased only when comparing pair- to singly-tested rats). Therefore, we propose that among the 14 call subtypes, it is specifically trill calls that are reward-associated. Flat calls, in contrast, have been observed in social conditions that are not necessarily appetitive (Burgdorf et al. 2008a; Stevenson et al. 2009; Wohr et al. 2008). In the present study, flat calls tended to be more prevalent in singly-tested rats than pair-tested rats, consistent with the proposal that their purpose is to re-establish social contact (Wohr et al. 2008). We also found a negative correlation between flat calls and trills, further suggesting that they are functionally distinct.

Conclusion

Our new classification scheme represents a significant extension of previous work by others, but it is unlikely to be complete. For example, only future investigation will show whether it generalizes to adult male rats in other behavioral or pharmacological test conditions, female rats, other rat strains, and other stages of adult development. It is natural to speculate that the various individual call types each communicate unique information. Hence, in the short term, it will be important to try to elucidate the behavioral significance of 50-kHz call subtypes, for example by identifying experimental conditions that preferentially elicit them, and by studying the effects of call subtype playback on rodent behavior. If the meaning of 50-kHz call subtypes can be deciphered, this may offer new avenues for future behavioral and pharmacological studies.

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