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Ultrasonic Vocalizations of Rats (*Rattus norvegicus*) During Mating, Play, and Aggression: Behavioral Concomitants, Relationship to Reward, and Self-Administration of Playback

Jeffrey Burgdorf, Roger A. Kroes, and Joseph R. Moskal
Northwestern University

James G. Pfaus
Concordia University

Stefan M. Brudzynski
Brock University

Jaak Panksepp
Northwestern University and Washington State University

Rats (*Rattus norvegicus*) emit a variety of ultrasonic vocalizations throughout their lifespan that reflect different forms of emotional arousal and accompanying affective states. In this study, high frequency recordings of ultrasonic vocalizations were made during mating, aggression, and both conspecific and heterospecific (dubbed "tickling") rough-and-tumble play behavior. We found that frequency modulated 50-kHz calls (trills and step calls) were positively correlated with positively valenced appetitive behavior during mating, play, and aggression. These calls were also positively correlated with the reward value of these social encounters. However, constant frequency (i.e., flat) 50-kHz calls were not related to appetitive behaviors or reward. In contrast, 22-kHz calls were positively related to aversive/withdrawal behaviors during mating, play, and aggression. Finally, we found that rats self-administered playback of frequency modulated 50-kHz trill calls and avoided playback of 22-kHz calls. Playback of flat 50-kHz calls or tape hiss was neutral. These results suggest that frequency modulated 50-kHz calls index a positively valenced, appetitive, social-emotional state in rats.

Keywords: rats, emotion, ultrasonic vocalizations, 50-kHz calls, 22-kHz calls

Adolescent and adult rats (*Rattus norvegicus* in the present work) emit ultrasonic vocalizations (USVs) in a wide variety of social encounters (Knutson, Burgdorf & Panksepp, 2002) including, from highest to lowest 50-kHz vocalization rate: rough-and-tumble play (Burgdorf, Panksepp, Beinfeld, Kroes, & Moskal, 2006; Knutson, Burgdorf & Panksepp, 1998), mating (McGinnis & Vakulenko, 2003; White, Cagiano, Moises, & Barfield, 1990), and aggression (Thomas, Takahashi, & Barfield, 1983; Vivian & Miczek, 1993). USVs have been hypothesized to serve as an adaptive social-communication signal for rats (Brudzynski, 2005; Brudzynski & Holland, 2005; Knutson et al., 2002), given that many predators cannot hear ultrasonic calls, and such vocalizations attenuate rapidly in natural environments (Nyby & Whitney, 1978). Studies in which rats were devocalized prior to social encounters demonstrated modest deficits in social interaction, which could be ameliorated by the playback of USVs (White & Barfield, 1987, 1989, 1990). Thus, USVs also appear to help facilitate social behavior in laboratory rats. In preliminary work, we have found that young rats prefer to spend more time with adults that emit abundant 50-kHz USVs than with animals that emit few such calls (Panksepp, Gordon, & Burgdorf, 2002).

Adolescent and adult rats emit two classes of ultrasonic vocalization: 50-kHz USVs that reflect high levels of behavioral arousal and positively valenced appetitive motivation, and 22-kHz USVs that reflect low levels of behavioral activation and the presence of an aversive motivational state (Brudzynski, 2007; Knutson et al., 2002). During rat aggression, mating, and rough-and-tumble play behaviors, rates of 50-kHz USVs decrease and rates of 22-kHz USVs increase from the beginning to the end of a single test session that mirrors the transition from appetitive behaviors to avoidance behaviors that occur across a single test session (Burgdorf, Wood, Kroes, Moskal, & Pankseep, 2007; Panksepp, Burgdorf, Beinfeld, Kroes, & Moskal, 2004; White et al., 1990). Therefore, USVs may help coordinate social behaviors in both the initiation-maintenance phase as well as the termination phase of adolescent and adult social encounters in rats (Knutson et al., 2002), and mice (Panksepp et al., 2007).

In the present study rat USVs were compared across the primary social behaviors exhibited in adolescent and adult rats, namely, rough-and-tumble play behavior, aggression, and mating, to further understand the behavioral and motivational concomitants of

Jeffrey Burgdorf, Roger A. Kroes, and Joseph R. Moskal, Falk Center for Molecular Therapeutics, Department of Biomedical Engineering, Northwestern University; James G. Pfaus, Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University; Stefan M. Brudzynski, Department of Psychology and the Centre for Neuroscience, Brock University; Jaak Panksepp, Falk Center for Molecular Therapeutics, Department of Biomedical Engineering, Northwestern University, and Department of Veterinary and Comparative Anatomy, Physiology, and Pharmacology, College of Veterinary Medicine, Washington State University.

Correspondence concerning this article should be addressed to Jeffrey Burgdorf, Falk Center for Molecular Therapeutics, 1801 Maple Avenue, Suite 4300, Evanston, IL 60201. E-mail: j-burgdorf@northwestern.edu

these vocalizations. USVs were recorded via direct high frequency recordings (Burgdorf et al., 2007; White et al., 1990) that allow for the detection of ultrasonic calls that cannot be reliably quantified from frequency-transformed recordings obtained from a bat detector. Frequency transformed recordings cannot readily distinguish between flat and frequency modulated 50-kHz USVs. In these experiments, we sought to quantify USVs recorded without transformation from a bat detector, which were emitted during behavioral episodes of aggression, sexual behavior, and rough-and-tumble play. We also correlated these USVs with the quantities of the aggressive, sexual, and play behaviors exhibited during these social interactions.

Thus, three experiments were performed. Experiment 1 was designed to determine if the flat and/or frequency modulated 50-kHz USVs (bandwidth of >18-kHz, and < 20 kHz, respectively) would correlate with positive appetitive behaviors (e.g., chasing during mating) while flat or frequency modulated (FM) 22-kHz USVs (van der Poel & Miczek, 1991) would be correlated with aversive behaviors (e.g., submissive freezing behavior; Knutson et al., 2002). Experiment 2 was designed to test the hypothesis that FM 50-kHz USVs (a subpopulation of all 50-kHz calls) would be positively correlated with the rewarding value of play behavior. Based on the results of Experiments 1 and 2 showing that FM 50-kHz USVs are related to reward and positive appetitive behavior and 22-kHz USVs were related to aversive behavior, Experiment 3 was designed to test the hypothesis that rats would self-administer playback of the FM 50-kHz USVs, and avoid playback of 22-kHz USVs. The work was conducted at two universities as indicated below.

Method

Animals and Housing

BGSU

Long Evans rats (*Rattus norvegicus;* Harlan Sprague dawley, Indianapolis, IN) born and bred in the Bowling Green State University (BGSU) animal facilities were used in these studies (Experiments 1 to 3). All animals used in these three studies were weaned at 21 days of age and singly housed except for the resident animals in the aggression study that were pair housed with tubal ligated females (purchased from Harlan Sprague dawley, Indianapolis, IN), 2 weeks before the start of testing at 3 months of age. All animals were housed in 20 × 40 × 20 cm translucent polycarbonate cages with corncob dust-free bedding. All animals were used in just one experiment. Experimental protocols were approved by the BGSU Institutional Animal Care and Use Committee.

Brock University

In Experiment 2, socially housed (3 per cage) or isolate housed from weening (at 21 days of age) adolescent Long Evans rats (Harlan Sprague dawley, Indianapolis, IN) were born and bred at the Brock University animal facilities and housed in 26.7 × 24.1 × 20.3 cm translucent polycarbonate cages with a similar corncob dust-free bedding as used at BGSU. Subjects were maintained on a 12:12 light–dark cycle (lights on 8:00 a.m.), and were given ad libitum access to Purina lab chow and tap water throughout the study. All animals were used in just one experiment. Experimental protocols were approved by Brock University Institutional Animal Care and Use Committee.

Ages and Housing Conditions

For the experiments involving heterospecific and conspecific play (Experiments 1 & 2), male isolate-housed adolescent rats aged 29 to 39 days were initially used given that animals isolate housed from weaning tested at these ages show the maximal amount of rough-and-tumble play behavior (Panksepp, 1981). Rates of heterospecific play (i.e., "tickling")-induced USVs were also analyzed from group housed (3 to 5 per cage) and singly housed male adolescent rats (29 to 34 days of age). Males were used in these adolescent studies because at these ages males emit higher rates 50-kHz USVs during heterospecific play compared to females (Panksepp & Burgdorf, 2003).

Six-month-old isolate-housed female rats were tested for heterospecifc play-induced USVs and reward. By 6 months of age, isolate-housed rats show marked decrease in tickle-induced USVs compared to adolescent (~1-month-old) and young adult rats (~2 to 3-month-old; Panksepp & Burgdorf, 2003). Six-month-old female rats were chosen for this study because at this age females emit higher rates of 50-kHz USVs during tickling than males (Panksepp & Burgdorf, 2003). Animals in this study were separated into tickle responders, which did not show an age related reduction in 50-kHz USVs, and tickle nonresponders that did show an age related reduction in 50-kHz USVs. This division represents approximately the upper and lower quartiles of 50-kHz USVs in 6-month-old isolate-housed female rats.

For the mating and aggression studies (Experiment 1), 3-month-old isolate-housed male rats were used to better compare the results of these studies with other published studies on mating/aggression and vocalizations (e.g., Thomas et al., 1983), and the results of the other experiments performed in the present series of studies.

Ultrasonic Vocalization Recording and Analysis

USVs were recorded from the high frequency output of a Pettersson D980 bat detector and condenser microphone (Pettersson Elektronik AB, Sweden). Thus, the signal was not transformed by the bat detector (i.e., as in frequency division or heterodyne recordings). High frequency output was directly recorded with a Fostex FR2 field recorder (Boonton, NJ) at a 192-kHz sampling frequency onto a 1 GB compact flash card as a .wav sound file (SanDisk, Milpitas, CA). Sonograms were produced using SAS Lab Pro (Avisoft Bioacoustics, Germany) on a personal computer. Sonograms were scored manually from the computer screen with a hand counter. All USVs were scored blind with respect to the additional nonvocal behavioral data.

During the rough-and-tumble play study in Experiment 2, USVs were recorded during play sessions and scored from sonograms using 1/10 frequency division output of a Pettersson D980 bat detector (Pettersson Elektronik AB, Sweden).

Experiment 1

Mating Behavior

Five 3-month-old male and five 3-month-old female rats were used in this study; each were individually housed. The experimen-

tal mating protocol was similar to Kippin and Pfaus (2001). Females received oviduct ligation surgery by Harlan (Harlan Sprague Dawley, Indianapolis, IN) before arrival at the BGSU animal facility. As described by Harlan, two bilateral incisions were made on the dorsal surface of the rats, each oviduct is exteriorized through the muscle wall and double ligated, and the incisions were closed with wound clips. Oviduct ligated females ($n = 5$) were made sexually receptive by subcutaneous injections of estradiol (10 μg; Sigma Aldrich, Saint Louis, MO) followed 48 hr later by progesterone (500 μg; Sigma Aldrich, Saint Louis, MO), and mating sessions occurred 6 hr after progesterone administration. The mating chamber consisted of a Plexiglas cage (35 × 26 × 18 cm high) with a vertical divider (30 × 0.5 × 20 cm high) placed in the middle of the test chamber that allowed both the males and females to escape from each other (i.e., paced mating as described by Kippin & Pfaus, 2001). Before testing, both males and females were habituated in male–female pairs to the test chamber for two 30-min sessions. During habituation, males and females had free access to each other, however, no hormonal priming was given to the female. Testing, following hormonal priming, consisted of a single 60-min session that occurred during the first half of the animals' dark cycle. The standard mating behaviors for this paradigm were scored. Mounts, ejaculations, and postejaculatory interval (i.e., time between ejaculation and subsequent mount) were scored from video recordings taken from the long side of the mating chamber (Kippin & Pfaus, 2001). During mating both males and females produce both types of 50-kHz USVs (Barfield & Thomas, 1986), therefore, rates of 50-kHz USVs could be attributed to either animal, whereas 22-kHz USVs in this assay are exhibited almost exclusively by the male rat, as indexed by thoracic compression analysis (Panksepp et al., 2007; data not shown), and therefore the rates of 22-kHz USVs can be attributed to the behavior of the male rat. The frequency of each behavior, and the time at which the behavior occurred, were scored by a trained observer who was blind to the vocalization data.

Aggression

Eight adult male pairs were used in this study. Resident animals were 3 months of age and were pair housed with 3-month old oviduct-ligated females. Intruder males were 2 months of age and were isolate housed. All testing was conducted in a separate test room so that the social activities would not be disruptive to the rest of the colony. Approximately 30 min before testing, the female was removed from the "resident" male's cage. Social aggression testing consisted of placing the intruder male into the home cage of the resident animal for 30 min under dim (~2 Lux) light. A plastic lid was placed on top of the resident's home cage with a small hole (6 × 6.5 cm) to position the ultrasonic microphone ~5 cm above the plastic lid (i.e., approximately 25 to 30 cm from the rat). Only pairs in which intruders received at least 3 bites (the clearest index of aggression), with the first bite occurring within the first 5 min, were used for the 2 min post-bite analysis ($n = 6$). From these pairs, the vocalization pattern from the first bite was used for analysis, so as to be minimally confounded by the potential vocalization effects of previous bites and to capture vocalizations before and after the start of aggression. The intruder animals in the resident-intruder aggression test have been shown to produce the majority (~90 to 100%) of the ultrasonic vocalizations (Thomas et al., 1983) and hence these aversion-indicating USVs were assumed to all come from the intruders. This was verified by observing which animal exhibited rhythmic thoracic compressions that corresponded to each USV. For correlational analysis, freezing and bites received by the intruder animal were compared to rates of USVs. In our tests, intruders exhibited 97% of the freezing and received 100% of the bites. Dorsal contacts were exhibited by both experimental and resident animals.

Videotape records were manually scored for three behaviors: (a) dorsal contacts, (b) bites (all of which were accompanied by audible squealing by the intruder), and (c) the subsequent freezing behavior. As noted, bites were exclusively received by the intruder male, and 22-kHz USVs and exclusively emitted by the intruder males. A freezing bout was counted after ~5 consecutive seconds without movement (except sniffing). Total duration of freezing by each intruder was used for subsequent analyses. Dorsal contacts (which in juvenile rats are elements of positive appetitive play behavior; Panksepp, Siviy & Normansell, 1984) consisted of one animal having both its front paws on the dorsal surface of the other animal. During aggression, dorsal contacts occur before the start of aggression and at rates that are ~70 times less than those during vigorous rough-and-tumble play. Dorsal contacts and bites were scored as frequency of occurrence for each animal. Other behaviors that commonly occur during sustained aggression (i.e., persistent on-top and submissive supine postures) did not occur frequently in these studies, probably because of the short periods of testing and perhaps due to the relatively low test cage height (20 cm). In the present study, the intruder rats were not significantly injured by the residents (i.e., no visible cuts and/or bleeding were seen in any animals).

Conspecific Play Behavior

Before the start of testing, animals were weaned (at 21 days of age) and assigned to play pairs matched by litter, sex, and weight at 23 days of age (i.e., brother–brother pairings). All animals ($n = 23$ pairs) received 5-min play sessions in the play chamber on the 2 days preceding testing, which served as habituation sessions. Each play test consisted of a single 30-min session in a 31 × 31 × 32 cm high box with corncob bedding under dim red light. Animals were 35 days of age at testing, and play behavior was recorded with a commercially available video camera (AG–DVC30, Panasonic, Secaucus, NJ) and DVD recorder (DMR–T6070, Panasonic, Secaucus, NJ), and high frequency USVs were recorded as in the previous experiments (Burgdorf et al., 2007). The frequency of occurrence as well as duration of dorsal contacts (one animal having both its front paws on the dorsal surface of the other animal) and pins (one animal laying in supine posture with the other animal above the pinned animal) were scored for both animals by a trained observer (Panksepp et al., 1984).

Experiment 2

Heterospecific Play (i.e., "Tickling") and Conspecific Play Behaviors

Heterospecific play (dubbed "tickling") consisted of vigorous whole-body playful tactile simulation with a human hand that included repeated pinning of the animal. For all animals, the

tickling was done with one hand and consisted of scaled-down rapid finger and hand movements on the pinned animal, commonly used in human tickling, with the sequence of events resembling rough-and-tumble play of juvenile rats. Methods and experimental design were identical to those in the study by Burgdorf and Panksepp (2001). In brief, tickling was conducted in a 45 × 35 × 20 cm high opaque plastic box without bedding. Even though the tactile stimulation was rapid, brisk, and assertive, care was taken not to threaten the animals. Before testing, all animals received one 2-min tickling session that served as habituation on the day preceding testing. After some experience with tickling, most animals spontaneously approached and followed the experimenter's hand. Three experiments were run.

Experiment 2a. Approach latency data was collected by placing the rat in one corner facing the palm of the experimenter's hand, which was situated in the corner diagonal to the rat (distance about 50 cm). The latency in seconds for the rat to approach and touch the experimenter's hand with its head or at least one front paw was recorded with a digital timer (to 1 s). The maximum approach latency was set at 30 s. These animals were 29 to 34 days of age during testing ($n = 14$ isolate-housed animals, $n = 18$ pair-housed animals).

Experiment 2b. Isolate-housed adult animals were 6 months old during testing, and were chosen from a larger pool of rats ($n = 68$) based on tickle-induced USVs at 3 months of age (as described above). Tickle responders ($n = 9$) represented the upper quartile of animals exhibiting tickle-induced 50-kHz USVs and tickle nonresponders ($n = 9$) represented the lower quartile of this sample population.

Experiment 2c. Nine 1-month-old juvenile isolate-housed play pairs were used in the conspecific play-induced place preference study. All animals were habituated at 36 days of age to the two-chamber unbiased conditioned place preference apparatus (30 × 60 × 30 cm) with the divider (20 × 15 cm) removed to allow access to both chambers for 15 min on the day prior to the start of testing (Burgdorf, Knutson, Panksepp, & Shippenberg, 2001). Conditioning consisted of 3 consecutive days of allowing each pair to play for the first 15 min on the white side, and then being moved by the experimenter to the black side for the second 15 min of play. Place preference testing occurred on the day after the end of the last conditioning session and consisted of allowing the animals to explore both the black and white sides of the place preference chamber (divider removed) for 15 min.

Experiment 3

Apparatus for Self Administrations of Playback of Ultrasonic Vocalizations

An unbiased operant sound-sample self-administration chamber was used in this study. The apparatus consisted of a 30 × 30 × 50 cm Lucite box with two holes (each 5 cm from the floor, 3.1 cm in diameter) on opposing walls. Nosepoking by rats broke a photobeam, which, with the aid of a personal computer, automatically counted the frequency and duration of each nosepoke. Nosepokes in the active hole elicited playback of a tape loop of a single exemplar of each USV type from high frequency recordings played back via a Fostex FR2 field recorder (Fostex, Boonton, NJ) into a preamplifier and speaker (speaker's working range was 1 to 120 kHz; Avisoft Bioacoustics, Germany) that was placed on top of the operant box ceiling with a 10.5 cm hole drilled out to accommodate the speaker. Animals were situated ~50 cm from the loudspeaker. USV playback lasted as long as the animal continued to nosepoke in the active hole, whereas nosepokes in the inactive hole did not elicit playback. USVs used for these studies were recorded during rat mating test, the resident-intruder aggression test, and sessions consisting of conspecific and heterospecific rough-and-tumble play behaviors.

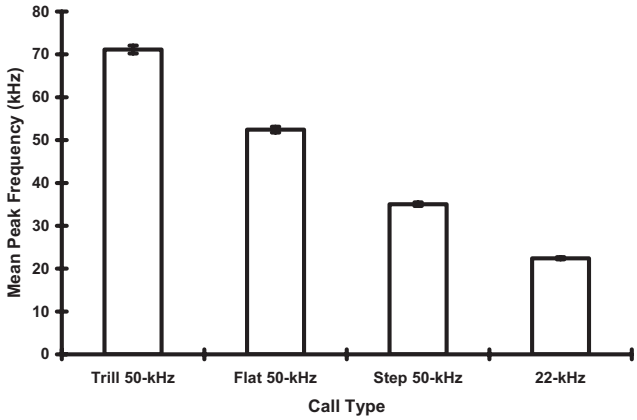

Figure 1. Mean (± 95% CI) peak frequency of constant frequency 50-kHz calls (flat 50 kHz), 22-kHz calls, as well as the trill and step components of FM 50-kHz calls. All recordings were direct (i.e., not transformed via a bat detector). The first 10 calls of each subtype were used from five pairs of animals tested during mating. All groups are significantly different from each other ($ps < .0001$).

On the day before the start of training, animals were habituated to the apparatus for 15 min, in which number and duration of baseline free operant nosepokes were recorded. On the three successive counterbalanced days of training, animals received either playback of a tape loop of an exemplar single (a) constant frequency 50-kHz USVs, (b) frequency modulated trill 50-kHz USVs, (c) constant frequency 22-kHz USVs, (d) FM 22-kHz USVs (as depicted in Figure 1), or (e) tape hiss in response to active hole nosepokes. Data from the last day of training and the habituation day were used for statistical analysis. Ultrasonic playback of the different call types was matched for intensity of background noise of the recordings (with 22-kHz USVs at approximately 85 dB from ~50 cm from the loudspeaker) and for percentage of time vocalizing.

Results

Sonographic Patterns of USVs

What has been traditionally referred to as 50-kHz calls (range of ~35 to 75 kHz with a mean peak frequency ~ 55-kHz) can contain step and/or trill components that have their own unique fundamental frequencies (see Figure 1), and bandwidths. Given the complexity of these calls, we have subdivided the class of 50-kHz calls into two subcategories, namely flat 50-kHz calls and FM 50-kHz calls. Exemplar sonograms for each ultrasonic call type are presented in Figure 2. Flat calls only contain the flat 50-kHz call component. FM 50-kHz calls may also contain the flat 50-kHz

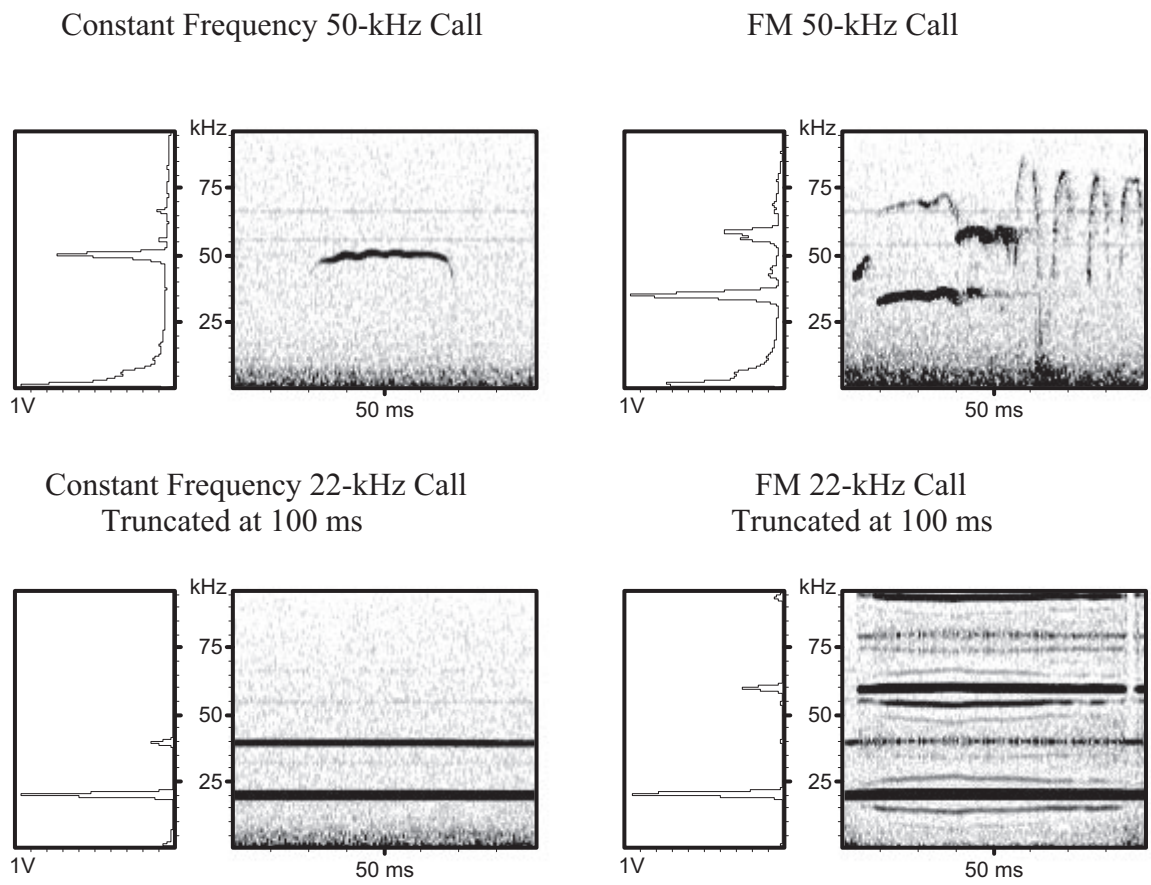

Figure 2. Sonographic examples of frequency modulated 50-kHz calls, flat 50-kHz calls, and 22-kHz calls. In the 22-kHz call depicted, the ~40-kHz harmonic may be an artifact of amplification. Although a flat-step-trill call is depicted as an exemplar of the FM 50-kHz call, the most common variant of FM 50-kHz calls are either a step-trill or a trill alone.

component, but must contain either the trill and/or step component. Less then 1% of all the recorded USVs did not fit into the flat 50-kHz, FM 50-kHz, or 22-kHz categories. Although not depicted, ~90% of the FM 50-kHz calls contained two or more components (most commonly, flat-trill, or flat-step). For further examples of frequency modulated 50-kHz calls, see White et al. (1990).

Experiment 1: Behavioral Concomitants of USVs During Mating, Aggression, and Play

Figure 1 shows the results of sonographic analysis of the step, trill, and constant frequency components of 50-kHz calls as well as 22-kHz calls. All peak frequencies for each call type were mutually exclusive distributions and significantly different from each other, $F(3, 196) = 1{,}153.8$, $p < .0001$, followed by a Fisher least significant difference (LSD) post hoc of all pairwise comparisons (all ps $< .0001$). Figure 3 shows the results of a histogram of the bandwidth of 50-kHz calls, which shows a bimodal distribution (failed a Kolmogorov–Smirnov normality test, $p < .0001$) that reflects non-frequency modulated (less than 18 Hz bandwidth) and frequency modulated USVs (greater than 20 kHz bandwith).

Figure 4 shows that rates of various USVs differ between rough-and-tumble play and aggression as indexed by a significant Testing Condition × Vocalization Subtype interaction, $F(2, 11) = 85.6$, $p < .0001$. FM 50-kHz USVs are significantly elevated and flat 50-kHz USVs and 22-kHz USVs are significantly reduced in rough-and-tumble play compared to aggression condition as indexed by a Fisher LSD post hoc comparisons of FM 50-kHz ($p < .0001$), Flat 50-kHz ($p < .05$), and 22-kHz calls ($p < .001$).

During mating tests, frequency rates of constant frequency (flat) 50-kHz, FM 50-kHz, and 22-kHz calls were compared 2 min before and 2 min after ejaculation, and differed significantly between vocalization subtypes, $F(2, 12) = 46.5$, $p < .0001$ (Figure 5A). Although the rate of the flat 50-kHz USVs remained unchanged 2 min before and after ejaculation (Fisher LSD post hoc, $p > .05$), FM 50-kHz USVs decreased (Fisher LSD post hoc, $p < .0001$), and 22-kHz USVs increased (Fisher LSD post hoc, $p < .001$; Figure 5B). During testing, mating pairs exhibited frequencies ($M \pm 95\%$ confidence intervals (CI) for the following behaviors; mounts (79.8 ± 14.4), ejaculations (2.0 ± 0.4), and latencies (in seconds) to the first incidence of the following behaviors, mounts (1.2 ± 1.2), ejaculation (708.0 ± 274.4), postejaculatory interval (840.2 ± 246.2).

During aggression testing, frequency counts of constant frequency 50-kHz, FM 50-kHz, and 22-kHz calls were compared 2 min before and 2 min after bites, and differed significantly between vocalization

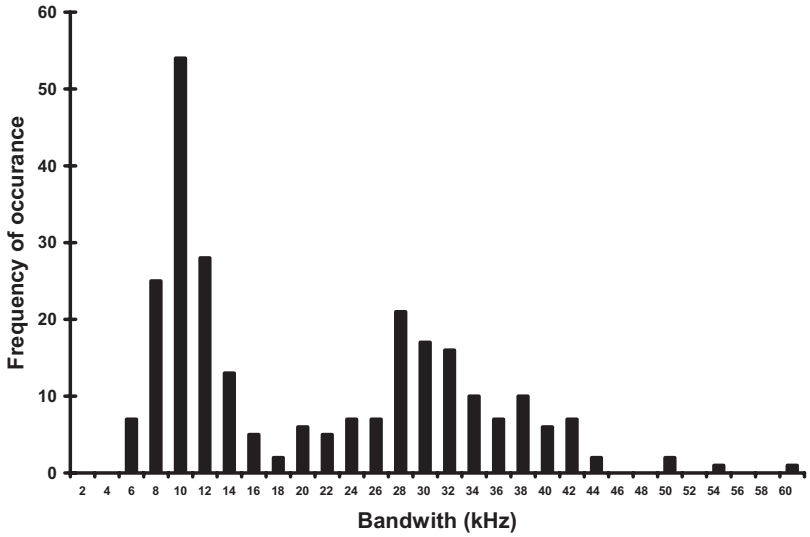

Figure 3. Bandwidth histogram of 50-kHz USVs that occurred during mating ($n = 2$), rough-and-tumble play ($n = 2$), and aggression ($n = 2$; total calls = 260). The first 30 to 50 calls were analyzed per pair with a similar number of calls per animal. Ultrasonic calls with a bandwidth range of 6- to 18-kHz calls are classified as nonfrequency modulated, and USVs with a bandwidth range of 20 to 60-kHz calls are classified as FM.

subtypes, $F(2, 15) = 7.9$, $p < .005$ (Figure 5B). FM 50-kHz and Flat 50-kHz calls decreased after bites (Fisher LSD post hoc, $ps < .01$), whereas 22-kHz calls increased after bites (Fisher LSD post hoc, $ps < .0001$; Figure 5B). For the statistical analysis, the first 15 incidents of 22-kHz calls, in which the presence or absence of movements could be clearly discerned, were used for each pair, $\chi^2(1, N = 531) = 55.3$ $p < .0001$ (Figure 6). In an analysis of that subset of postejaculation 22-kHz USVs, out of all the calls that occurred while the male rat was grooming or showing mouth movements (i.e., tongue protrusions), 96% were frequency modulated, whereas only 3% of the calls were frequency modulated when the male rat was not grooming or showing mouth movements.

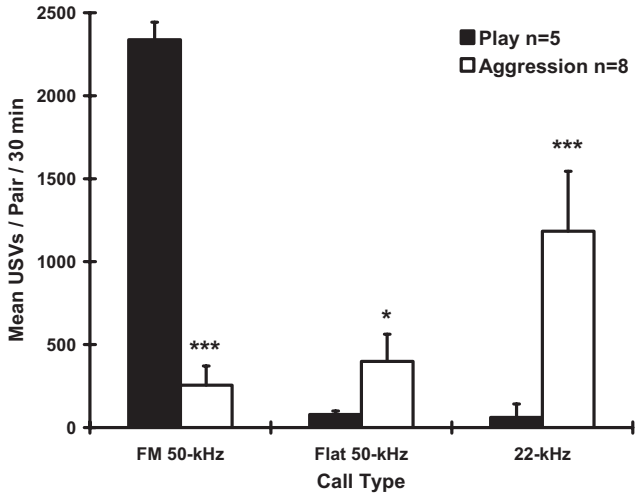

Figure 4. Mean (± 95% CI) of FM 50-kHz calls, flat 50-kHz calls, and 22-kHz USVs during 30 min of rough-and-tumble play ($n = 23$ pairs) at 1 month of age as compared to 30 min of aggression ($n = 18$ pairs) at 3 months of age. $^{*}p < .05$. $^{***}p < .001$, Fisher LSD post hoc test, two-tailed.

Correlations between rates of USVs and rates of approach (chasing and dorsal contacts) or avoidance associated behaviors that occur during mating, play, or aggressive behavior are summarized in Table 1. The largest effects were positive correlations between FM 50-kHz and all appetitive behaviors during play, sexual, and aggressive activities. The largest negative correlations were evident between FM 50-kHz USVs, and avoidance activities during these social interactions, including the flat 50-kHz USVs in relation to freezing during aggressive encounters. Strong positive correlations were seen between 22-kHz and avoidance behaviors during all three types of social activities.

Experiment 2: The Relationship Between USVs During Play and Reward

Experiment 2A

In adult female rats receiving tickling stimulation, animals previously shown to have high levels of 50-kHz calls in response to tickling (tickle responders) differed significantly in rates of USVs as indexed by a significant Vocalization Subtype × Responder/Nonresponder interaction, $F(2, 16) = 29.8$, $p < .0001$ (Figure 7A). Tickle responder animals showed significantly more FM 50-kHz calls (Fisher LSD post hoc, $ps < .0001$), but no change in constant frequency 50-kHz calls or 22-kHz calls than the nonresponder animals (all $ps > .05$; Figure 7A). Tickle responders also showed shorter approach latency to self-administered tickling than nonresponder animals, $F(1, 16) = 66.1$, $p < .0001$ (Figure 7B).

Experiment 2B

In adolescent animals, housing conditions (isolate vs. social) differentially affected rates of USVs subtypes compared to socially

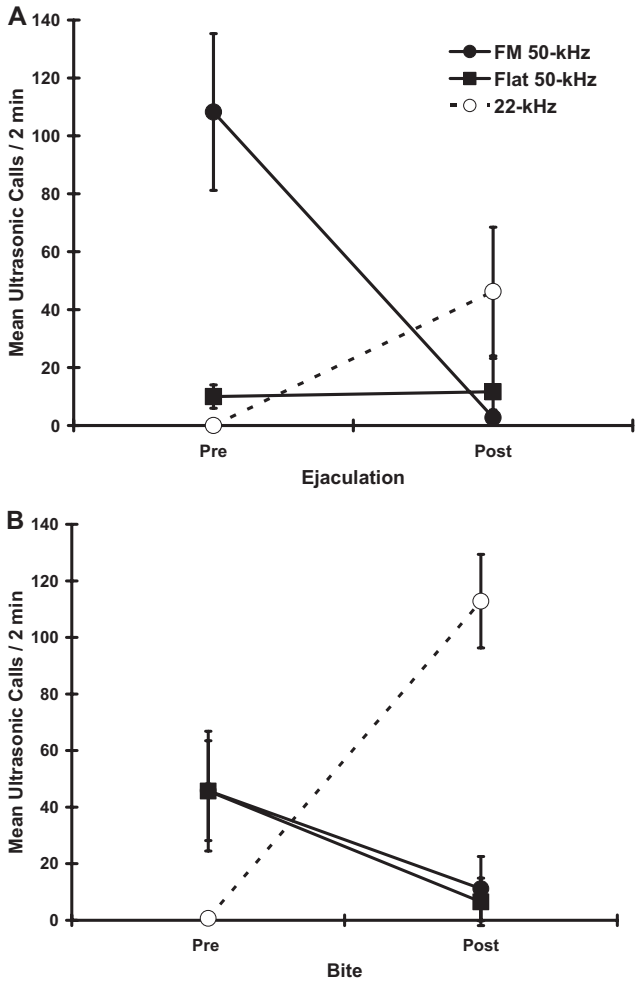

Figure 5. Mean (± 95% CI) number of ultrasonic calls in sexual and aggressive behavior. A: FM 50-kHz calls, flat 50-kHz calls, and 22-kHz calls 2 min before (pre) and the 2 min after (post) an ejaculation ($n = 5$) during mating at 3 months of age. B: FM 50-kHz calls and 22-kHz calls 2 min before (pre) and the 2 min after (post) a bite during intermale aggression ($n = 8$ pairs) at 3 months of age.

housed animals; Housing × Vocalization Subtype interaction, $F(2, 30) = 4.6$, $p < .05$. Isolate-housed animals produced more FM 50-kHz calls (Fisher LSD post hoc, $p < .05$) but not constant frequency (flat) 50-kHz or 22-kHz calls compared to socially housed animals ($p > .05$; Figure 8).

Experiment 2C

During conspecific rough-and-tumble play, rates of 50-kHz USVs, within-subjects $t(8)$ 3.5, $p < .01$; pinning behavior, within-subjects $t(8) = 4.7$, $p < .005$; and dorsal contacts, within-subjects $t(8) = 9.0$, $p < .001$ were greater during the first 15 min of play as compared to the last 15 min of play, whereas 22-kHz USVs did not significantly differ between the first and last 15 min of play ($p > .05$). Animals showed a significant place preference for the environment paired with the first 15 min of play as compared to the second 15 min of play, within-subjects $t(8) = 3.0$, $p < .05$. Correlations between the rewarding value of heterospecific play (as measured by approach latency in seconds) and conspecific play behavior (as measured by conditioned place preference in seconds) and rates of USVs (FM 50-kHz, flat 50-kHz, and 22-kHz) are reported in Table 2. These results show that FM 50-kHz USVs are positively correlated with the rewarding value of play, whereas no significant correlation are seen between rates of flat 50-kHz or 22-kHz USVs and the rewarding value of play.

Experiment 3: Self-Administration of Playback of USVs

As depicted in Figure 9, during the 15-min habituation session, animals showed no preference for the hole designated to be "active" during testing. Nosepoking of the active hole differed significantly across the playback conditions, $F(3, 62) = 8.0$, $p < .001$. Playback of FM 50-kHz calls was the only vocal pattern that led to significantly increased nosepoking as compared to the habituation sessions (Fisher LSD post hoc, $p < .005$), and it was also higher than any of the other playback conditions. Active hole nosepoking was clearly decreased by playback of 22-kHz USVs (Fisher LSD post hoc, $p < .0001$). No significant change was evident in the flat 50-kHz group or tape hiss control group (all ps $> .05$; Figure 9).

Discussion

The present results demonstrate that the FM subtype of 50-kHz calls is related to positive appetitive behavior during rough-and-tumble play behavior and mating (see Table 1) and is positively correlated to the rewarding value of the vocalization eliciting stimulus during both conspecific and heterospecific play (see Table 2). The conditioned approach latency and conditioned place preference measures further confirmed the association between 50-kHz USVs and reward. These findings do not support the hypothesis that these USVs are artifactual byproducts of locomotor arousal (Knutson et al., 2002). FM 50-kHz USVs may reflect a positive affective state that accompanies both social and nonsocial rewards because the FM 50-kHz USVs variant indexes drugs and brain reward, and these calls are modulated by the mesolimbic dopamine system (Burgdorf et al., 2007; Ikemoto & Panksepp, 1999). These data are consistent with previous studies demonstrating that 50-kHz calls are associated with positively valenced appetitive behaviors and reward (Knutson, Burgdorf & Panksepp, 2002; McGinnis & Vakulenko, 2003). These results corroborate a previous finding of a type of vocalization that is specific to both social and nonsocial reward in the mongoose (Rasa, 1984).

Although it remains unclear what specific function flat 50-kHz USVs may have in regulating social behavior, the results of the current study suggest that the flat calls are more evident in aggressive situations than positive social interactions, and may index social ambivalence. Aggressive encounters were accompanied by much higher percentages of flat 50-kHz calls as compared to FM calls, and the only significant relationship to behavior found for flat 50-kHz calls was a negative correlation with submissive behavior of the defeated animal. A recent study showing that rats in nonrewarding social interactions exhibit far more flat 50-kHz USVs compared to FM 50-kHz USVs suggesting that the flat 50-kHz USVs may serve as a nonaffective contact call (Wöhr, Houx, Schwarting, & Spruijt, 2008).

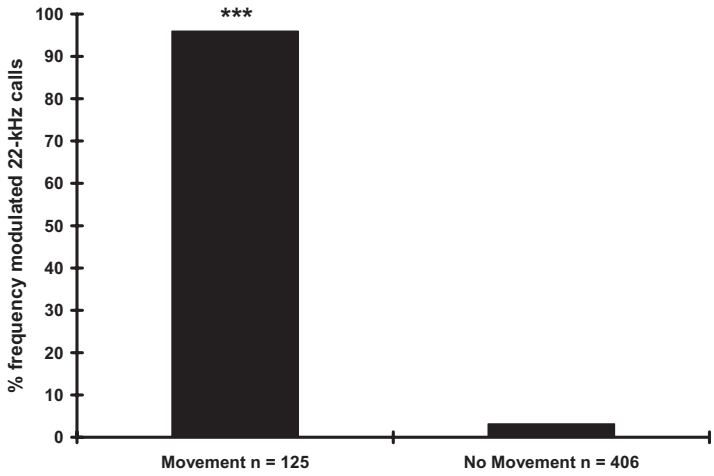

Figure 6. Percentage of postejaculatory calls that were frequency modulated while the animal was engaging in grooming or tongue protrusions (movement group) during vocalizing or was not grooming (no movement group). The first 15 22-kHz ultrasonic calls in which mouth movement of the male could be clearly observed was used from five mating pairs. *** $p < .001$, chi square test, two-tailed, comparing movement versus no-movement groups.

The presence of 50-kHz USVs during aggression has been suggested to negate our conclusion that 50 kHz calls reflect a positive affective state (Berridge, 2003). We now show that aggressive encounters had a much higher percentage of flat 50-kHz calls as compared to FM 50-kHz calls, and the only significant relationship to behavior found for flat 50-kHz calls was a negative correlation with submissive behavior of the defeated animal. In addition, it was evident in the present work, as well as in previously published studies (Panksepp & Burgdorf, 2003), that 50-kHz USVs occur primarily before the start of aggression, and are greatly reduced after clear aggressive behavior (i.e., biting) has occurred.

These results also demonstrate that the FM 50-kHz calls can be empirically separated from flat 50-kHz calls basis of the average peak frequency of the call (see Figure 1) and bandwidth (see Figure 3). Rats appear to space the peak frequency of their subtypes of ultrasonic vocalization in relatively even steps across their ultrasonic hearing range (see Figure 1) which extends up to 80 kHz (Kelly & Masterton, 1977).

Table 1
Correlations Between the Rewarding Value of Play and USVs

	Appetitive/Approach Behavior		
Call Subtype	Mating (Chases)	Play (DC)	Aggression (DC)
22-kHz	–.30*	–.12	–.31*
Flat 50-kHz	–.22	–.14	+.13
FM 50-kHz	+.63**	+.59**	+.35*
Call Subtype	Mating (PEI)	Play(Pin Duration)	Aggression (Freezing)
22-kHz	+.53**	+.41*	+.69**
Flat 50-kHz	–.19	+.35	–.70**
FM 50-kHz	–.57**	–.68**	–.69**

Note. Spearman's correlations were used for all comparisons involving 22-kHz calls and PEI during mating, and Pearson's correlations were used for all other comparisons. USVs = ultrasonic vocalizations; DC = dorsal contacts; FM = frequency modulated; PEI = postejaculatory interval.
* $p < .05$. ** $p < .005$.

This work also revealed that FM components of the 22-kHz USVs that occur after ejaculation may reflect grooming or tongue protrusion artifact (see Figure 6). Given that frequency modulated 22-kHz USVs have been found to occur in greater number during mating than aggression (van der Poel & Miczek, 1991), it is possible that FM 20-kHz USVs may reflect different affective states in the sender. It is also possible that the patterns of tongue protrusions and grooming that occur after mating resembles those seen during postprandial grooming (Berridge & Fentress, 1986). However, further studies involving slow motion analysis comparing postprandial to postejaculatory grooming are required to test this hypothesis. The 22-kHz USVs that occur after ejaculation are thought to enforce the refractory period of the male rat and to perhaps deter any other males from mating with the target female during intromission (Barfield & Thomas, 1986), and was shown to facilitate paternity (Coria-Avila, Pfaus, Hernandez, Manzo, & Pacheco, 2004). Future studies involving the playback of these USVs are needed to examine the role of the subtypes of 50-kHz calls in regulating the socially rewarding and nonsocial reward behaviors of the rats.

There is now converging evidence across mating, rough-and-tumble play, and aggression studies that rats use ultrasonic vocalizations to pace and coordinate their social behavior, with FM 50-kHz calls being involved in the initiation and maintenance of positive social behaviors, and 22-kHz calls being involved in the cessation or withdrawal from social activities (Knutson et al., 2002). Rats will self-administer playback of FM 50-kHz USVs and avoid playback of 22-kHz USVs (see Figure 9). The experiments performed in this study were limited by the use of isolate-housed animals. Long-term isolate-housing has been shown to produce deficits in social (Cooke, Chowanadisai, & Breedlove, 2000), emotional (Inagaki, Kuwahara, Kikusui, & Tsubone, 2005), and cognitive behaviors (Teather, Magnusson, Chow, & Wurtman,

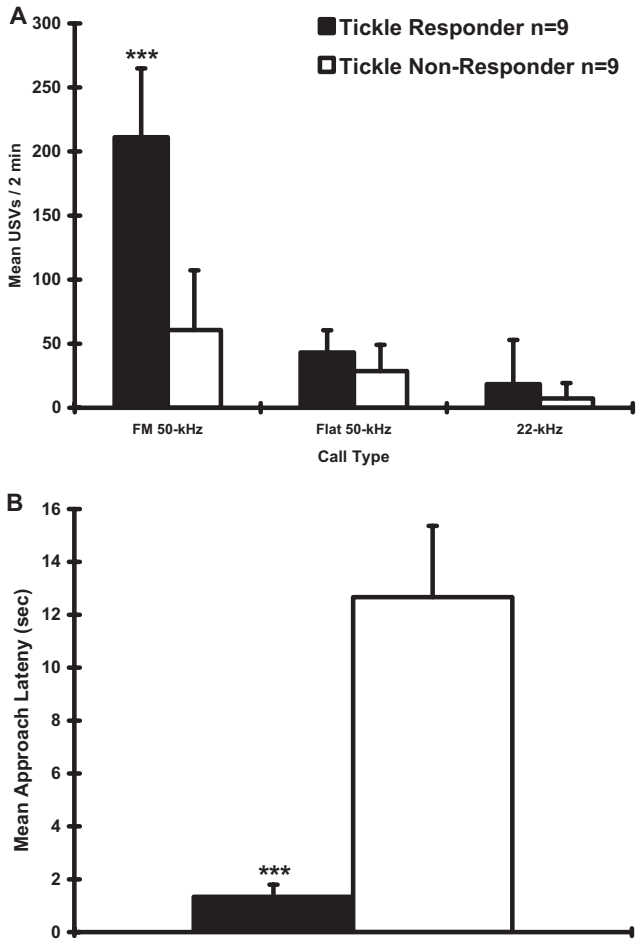

Figure 7. Mean (± 95% CI) number of ultrasonic calls and approach latency in heterospecific play of young rats. A: Ultrasonic vocalization rates in 6-month-old adult rats in response to 2 min of heterospecific play stimulation (i.e.,"tickling") in animals that during initial screening for vocalization responsivity were further subdivided into high ($n = 9$, tickle responders) and low ($n = 9$, tickle nonresponders) groups. B: Mean ± 95% confidence interval of approach latency for animals to self-administer additional tickling stimulation following the 2-min tickling test. $^{***}p <$ 001, analysis of variance, two-tailed.

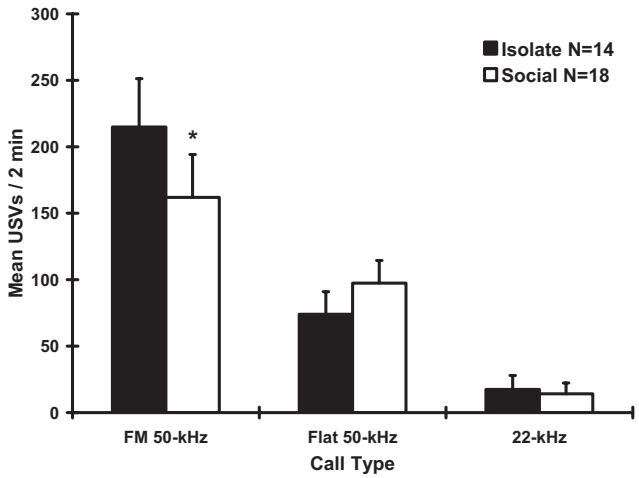

Figure 8. Mean (± 95% CI) ultrasonic calls in 1-month-old rats in response to 2 min of heterospecific play (i.e., "tickling") stimulation in animals housed alone (isolate) or in groups (social) for three types of calls: FM 50-kHz calls, flat 50-kHz calls, and 22-kHz calls. Previously, we have shown that adolescent isolate-housed animals find ticking to be more rewarding then social-housed animals (Burgdorf & Panksepp, 2001). $^{*}p <$ .05, Fisher LSD post hoc.

2002). Future studies using socially housed animals are required to further address these issues.

The neural mechanisms of 50-kHz and 22-kHz calls may shed light on the neural bases of positive and negative affective states in humans and other animals and have potential implications for understanding psychiatric disorders (Burgdorf et al., 2001; Brudzynski, 2007; Brudzynski, Eckersdorf, & Golebiewski, 1995; Panksepp et al., 2002). The use of vocal indicators of various other emotional states in other species has helped reveal emotional circuits that may be of importance in understanding the ancestral sources of human emotionality (Brudzynski et al., 1995; Jürgens, 2002; Newman, 1988; Panksepp, 2007). The analysis of the neuroanatomical substrates of separation calls in several mammalian species (Panksepp, Normansell, Herman, Bishop, & Crepeau, 1988) have highlighted brain areas that were eventually implicated in the genesis of human sadness (Damasio et al., 2000; Panksepp & Burgdorf, 2003). The powerful opioid control over separation-distress responsivity (Herman & Panksepp, 1978; Panksepp, Herman, Conner, Bishop, & Scott, 1978) fits well with the recent demonstration that human sadness is accompanied by low brain opioid tone (Zubieta et al., 2003).

Our hypothesis is that the robust tickle-induced FM 50-kHz calls in rats have some ancestral linkage to infantile human hedonic laughter (Panksepp, 2007; Panksepp & Burgdorf, 2003). This leads us to predict that when the executive neurochemical systems for this response are eventually identified in rats, they will also be found to regulate comparable positive affective states in humans. Although cross-species analysis of basic emotional systems has not been well accepted in academic psychology (e.g., Barrett, 2006), in-depth analyses of the neurochemical properties of such circuits in other animals can lead to novel testable predictions in

Table 2
Correlations Between the Rewarding Value of Play and USVs

Call Subtype	Tickling Reward	Play Reward
22-kHz USVs	–.04	+.09
Flat 50-kHz USVs	+.26	
FM 50-kHz USVs	+.60**	+.77*

Note. Heterospecific play ($n = 18$; i.e. "tickling") reward was measured by approach latency and rough-and-tumble conspecific play ($n = 9$ pairs; i.e., play) was measured by place preference. During conspecific play recordings, it was not possible to differentiate between flat 50-kHz frequency modulated 50-kHz USVs. However in Experiment 1 we found that 97% of all 50-kHz calls during conspecific play were frequency modulated (see Figure 4). Spearman's correlations were used for the reward 22-kHz comparison, and Pearson's correlations were used for all other comparisons. USVs = ultrasonic vocalizations.
$^{*}p < .05$. $^{**}p < .005$.

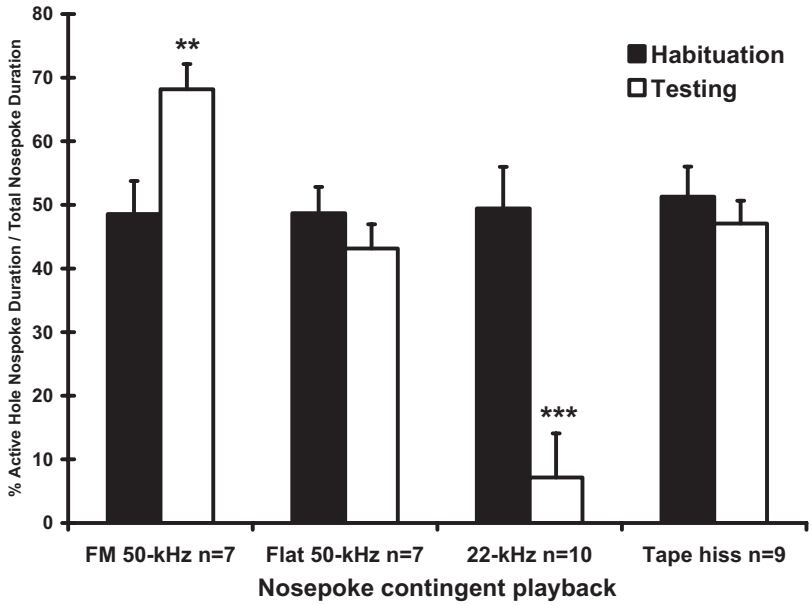

Figure 9. Mean (± 95% CI) nosepoke duration in the active hole compared to the inactive hole in groups of 3-month-old female rats. During habituation nosepokes in the hole that was to be activated during testing elicit no playback. During testing, nosepokes of the active hole elicited playback of FM trill 50-kHz, flat 50-kHz, FM 22-kHz, flat 22-kHz calls, or tape hiss. $^{*}p < .05$, within-subject t test (two-tailed) comparing active versus inactive hole. $^{**}p < 01$. $^{***}p < .001$, Fisher LSD post hoc.

our own species (Knutson et al., 2002; Panksepp, 1998, 2005; Panksepp & Harro, 2004).

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