Behavioral/Cognitive

Phasic Dopamine Release in the Nucleus Accumbens in Response to Pro-Social 50 kHz Ultrasonic Vocalizations in Rats

Ingo Willuhn,[1] Amanda Tose,[1] Matthew J. Wanat,[1] Andrew S. Hart,[1] Nick G. Hollon,[1] Paul E.M. Phillips,[1] Rainer K.W. Schwarting,[2] and Markus Wöhr[2]

[1]Departments of Psychiatry and Behavioral Sciences and Pharmacology, University of Washington, Seattle, Washington 98195 and [2]Behavioral Neuroscience, Experimental and Biological Psychology, Faculty of Psychology, Philipps-University of Marburg, 35032 Marburg, Germany

Rats emit ultrasonic vocalizations (USVs) that are thought to serve as situation-dependent affective signals and accomplish important communicative functions. In appetitive situations, rats produce 50 kHz USVs, whereas 22 kHz USVs occur in aversive situations. Reception of 50 kHz USVs induces social approach behavior, while 22 kHz USVs lead to freezing behavior. These opposite behavioral responses are paralleled by distinct brain activation patterns, with 50 kHz USVs, but not 22 kHz USVs, activating neurons in the nucleus accumbens (NAcc). The NAcc mediates appetitive behavior and is critically modulated by dopaminergic afferents that are known to encode the value of reward. Therefore, we hypothesized that 50 kHz USVs, but not 22 kHz USVs, elicit NAcc dopamine release. While recording dopamine signaling with fast-scan cyclic voltammetry, freely moving rats were exposed to playback of four acoustic stimuli via an ultrasonic speaker in random order: (1) 50 kHz USVs, (2) 22 kHz USVs, (3) time- and amplitude-matched white noise, and (4) background noise. Only presentation of 50 kHz USVs induced phasic dopamine release and elicited approach behavior toward the speaker. Both of these effects, neurochemical and behavioral, were most pronounced during initial playback, but then declined rapidly with subsequent presentations, indicating a close temporal relationship between the two measures. Moreover, the magnitudes of these effects during initial playback were significantly correlated. Collectively, our findings show that NAcc dopamine release encodes pro-social 50 kHz USVs, but not alarming 22 kHz USVs. Thus, our results support the hypothesis that these call types are processed in distinct neuroanatomical regions and establish a functional link between pro-social communicative signals and reward-related neurotransmission.

Key words: **dopamine; fast-scan cyclic voltammetry; nucleus accumbens; social behavior; ultrasonic communication**

Introduction

Rats emit various types of ultrasonic vocalizations (USVs). These calls are thought to serve as situation-dependent affective signals and accomplish important communicative functions (Portfors, 2007; Brudzynski, 2013; Wöhr and Schwarting, 2013). Three main types of USVs are typically distinguished. (1) Pups emit 40 kHz USVs ("distress calls") when socially isolated from mother and littermates, probably reflecting a negative affective state akin to anxiety (Vivian et al., 1997). (2) Juvenile and adult rats emit low-frequency 22 kHz USVs ("alarm calls") in aversive situations such as predator exposure (Blanchard et al., 1991), fear conditioning (Wöhr and Schwarting, 2008), or social defeat (Kroes et al., 2007). (3) High-frequency 50 kHz USVs ("rat laughter") occur in appetitive situations such as social investigation and rough-and-tumble play (Knutson et al., 1998) or upon administration of drugs of abuse such as amphetamine, either systemically (Ahrens et al., 2013; Wright et al., 2013; Pereira et al., 2014) or locally into the nucleus accumbens (NAcc; Burgdorf et al., 2001; Thompson et al., 2006).

In support of a communicative function, 50 and 22 kHz USVs induce call-specific behavioral responses in the receiver. The 50 kHz USVs induce social approach behavior in the recipient, indicating that they serve a pro-social function as contact calls (Wöhr and Schwarting, 2007, 2009, 2012); 22 kHz USVs lead to freezing behavior, suggesting an alarming function (Blanchard et al., 1991; Endres et al., 2007; Wöhr and Schwarting, 2007, 2008). These opposite behavioral responses are paralleled by distinct brain activation patterns as shown by means of electrophysiological recordings and immediate early gene expression: 22 kHz USVs induce activation in amygdala and periaqueductal gray (Sadananda et al., 2008; Parsana et al., 2012), both strongly impli-

Received March 17, 2014; revised June 6, 2014; accepted June 27, 2014.

Author contributions: I.W. and M.W. designed research; I.W., A.T., M.J.W., A.S.H., N.G.H., and M.W. performed research; I.W. and M.W. analyzed data; I.W., P.E.M.P., R.K.W.S., and M.W. wrote the paper.

This work was supported by grants from the Deutsche Forschungsgemeinschaft to R.K.W.S. (DFG 559/10-1) and to M.W. (DFG WO 1732/1-1), a National Institutes of Health grant to P.E.M.P. (R01-DA027858), and a National Science Foundation Graduate Research Fellowship to N.G.H.

The authors declare no competing financial interests.

Correspondence should be addressed to either of the following: Ingo Willuhn, Netherlands Institute for Neuroscience and Department of Psychiatry, Academic Medical Center, University of Amsterdam, 1105BA Amsterdam, The Netherlands, E-mail: ingo.willuhn@gmail.com; or Markus Wöhr, Behavioral Neuroscience, Experimental and Biological Psychology, Faculty of Psychology, Philipps-University of Marburg, Gutenbergstrasse 18, 35032 Marburg, Germany, E-mail: markus.woehr@staff.uni-marburg.de.

I. Willuhn's present address: Netherlands Institute for Neuroscience and Department of Psychiatry, Academic Medical Center, University of Amsterdam, 1105BA Amsterdam, The Netherlands.

DOI:10.1523/JNEUROSCI.1060-14.2014

 0270-6474/14/3410616-08$15.00/0

cated in the regulation of anxiety and fear (Fendt and Fanselow, 1999; LeDoux, 2000), whereas 50 kHz USVs inhibit the amygdala (Parsana et al., 2012) and evoke neuronal activation in the NAcc (Sadananda et al., 2008).

The NAcc is considered to be a limbic brain region that is strongly implicated in appetitive behavior. Specifically, the NAcc is thought to be critical for the translation of motivational processes into action by serving as an interface between limbic and motor systems in the brain (Mogenson et al., 1980). Engaging in appetitive actions depends on NAcc dopamine release from terminals of midbrain projections (Robbins and Everitt, 1982). For example, interference with dopamine neurotransmission impairs the ability to execute goal-directed behavior (Salamone et al., 1994). Furthermore, dopamine neurotransmission is known to encode the value of rewards (Schultz et al., 1997; Day et al., 2007). This role of phasic dopamine signaling in encoding environmental events with a positive valence led us to hypothesize that NAcc dopamine signaling is also involved in social approach toward pro-social 50 kHz USVs. Therefore, we tested whether 50 kHz USVs elicit phasic dopamine release in the NAcc using fast-scan cyclic voltammetry (Clark et al., 2010; Flagel et al., 2011; Willuhn et al., 2012).

Materials and Methods

Animals. Adult male albino rats from Charles River weighing between 300 and 450 g were housed individually and kept on a 12 h light/dark cycle (lights on at 0700) with controlled temperature and humidity. Rats ($n = 12$) were food deprived to 90% of their free-feeding body weight and water was provided *ad libitum*. All animal use was approved by the University of Washington Institutional Animal Care and Use Committee, and surgical procedures were performed under aseptic conditions.

Stereotaxic surgery. Rats were anesthetized with isoflurane, placed in a stereotaxic frame, administered with the nonsteroidal anti-inflammatory carprofen (5 mg/kg, subcutaneously), and placed on an isothermal pad to maintain body temperature. Before incision, the scalp was swabbed with alcohol and betadine, and treated with a mixture of lidocaine (0.5 mg/kg) and bupivacaine (0.5 mg/kg). Holes were drilled in the cranium and dura mater was cleared for targeting the NAcc (1.3 mm anterior, 1.3 mm lateral, and 7.2 mm ventral to bregma). A carbon-fiber microelectrode made in-house (Clark et al., 2010) was positioned in the NAcc, and an Ag/AgCl reference electrode was implanted in a separate part of the forebrain. Electrodes were secured with cranioplastic cement anchored to the skull by screws. Following surgery, carprofen (5 mg/kg) was administered again. Rats were monitored until ambulatory and were then given 1–2 weeks to recover from surgery.

Magazine training and unexpected food pellet delivery. To confirm that electrodes were capable of detecting dopamine, unexpected food pellets were delivered before playback sessions to elicit dopamine release. If the unpredicted delivery of a food pellet elicited an electrochemical response (positive control), voltammetry data form the playback session were analyzed. For this purpose, animals were placed in operant chambers ($32 \times 34 \times 34$ cm; Med Associates) that had grid floors and a house light and contained a food magazine. During two sessions of magazine training a total of 60 food rewards (45 mg of food pellets; Bio-Serve) were delivered at a 90 s variable interval. Between animals, operant chambers were cleaned with Roccal-D (1:250 dilution).

Playback of USVs—Acoustic stimuli. Dopamine release and behavioral responses to playback of USVs and acoustic control stimuli were tested under dim white light conditions in operant chambers placed inside a sound-isolation cubicle. Four acoustic stimuli (for review, see Wöhr and Schwarting, 2007, 2009, 2012), each with a duration of 15 s, were used. (1) Natural 50 kHz USVs (Fig. 1*A*), which had been recorded from an adult male Wistar rat during exploration of a housing cage with bedding containing scents from a recently separated cage mate. Playback calls were recorded in this context because playback of 50 kHz USVs recorded during tickling or exploration of an empty cage were reported to have no or only minor effects on recipient behavior (Burman et al., 2007; Endres et al., 2007). The 50 kHz USV stimulus consisted of a total of 58 individual 50 kHz USVs (total calling time: 3.89 s), together with background noise the sender had produced while exploring the cage. (2) Natural 22 kHz USVs (Fig. 1*B*), which had been recorded from a male Wistar rat exposed to electric footshocks. The 22 kHz USV stimulus consisted of a total of seven individual 22 kHz USVs (total calling time: 7.98 s). To control for background noise present in the 50 kHz USV playback, background noise was added to the 22 kHz USV playback. (3) Time- and amplitude-matched white noise (NOISE; Fig. 1*C*). Playback of NOISE was generated with the Avisoft SASLab Pro software (version 4.2, Avisoft Bioacoustics) by replacing 50 kHz USVs with white noise signals, with durations and amplitude modulations corresponding to that of the original 50 kHz USVs. Thus, the NOISE stimulus had the same temporal pattern as the 50 kHz USV stimulus and was identical to the original natural 50 kHz USV series with respect to all call features, apart from the fact that sound energy was not confined to a certain frequency. To control for background noise present in the 50 kHz USV playback, background noise was added to the NOISE playback. (4) Background noise (BACKGROUND; Fig. 1*D*). The BACKGROUND stimulus was presented to control for unspecific effects not linked to the communicative function of 50 kHz USVs, since the 50 kHz USV stimulus contained background noise, i.e., noises, which occur when a rat is exploring an arena with bedding. After 1 h of habituation, acoustic stimuli (50 and 22 kHz USVs, NOISE, and BACKGROUND) were presented through an ultrasonic speaker (ScanSpeak using the USG Player 116; Avisoft Bioacoustics) at ~69 dB (measured from a distance of 40 cm) with a sampling rate of 300 kHz in 16-bit format. Playback of acoustic stimuli was monitored by an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics).

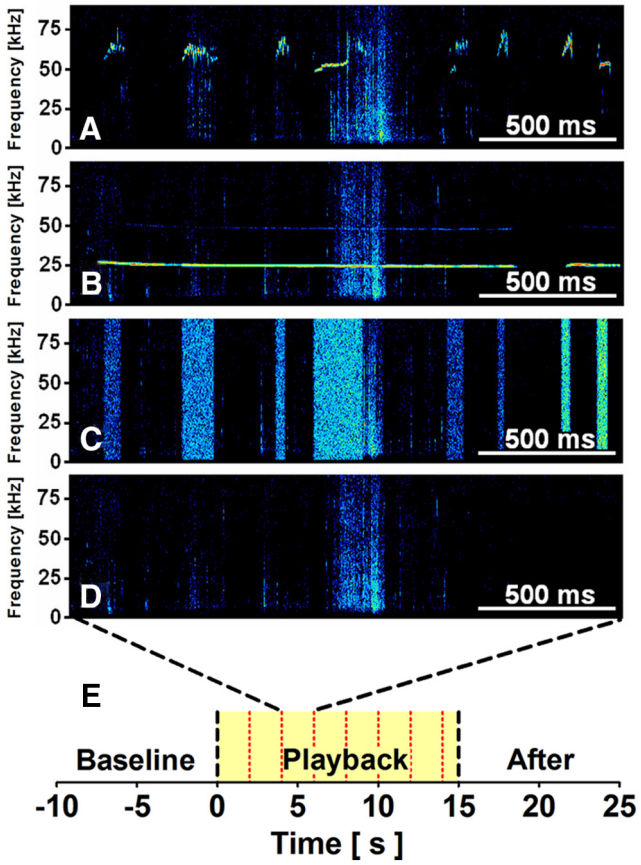

Figure 1. Acoustic stimuli used for playback. Exemplary spectrograms of (***A***) 50 kHz USVs, (***B***) 22 kHz USVs, (***C***) time- and amplitude-matched white noise (NOISE), and (***D***) background noise (BACKGROUND). ***E***, Acoustic stimuli were presented repeatedly for 15 s in random order by an ultrasonic loudspeaker (ScanSpeak; Avisoft Bioacoustics).

Playback of USVs—Procedure. Rats were tested individually. At the beginning of the experiment, all rats were exposed to NOISE to reduce novelty. Then, animals were repeatedly exposed to sequences of acoustic stimuli, with the four different acoustic stimuli presented in random order. Acoustic stimuli were presented for 15 s with an interstimulus interval lasting between 1 and 2 min (session duration: 1 h). Because this procedure resulted in ~40 presentations per session with at least six per acoustic stimulus, we restricted our analysis to these first six stimuli to maintain sample size between stimuli. A second session was conducted ~1 week after the first one. Dopamine release and behavior were assessed during the 15 s playback phases as well as for 10 s before and after playback (Fig. 1*E*). TTL signals were used for synchronizing playback of acoustic stimuli and voltammetric measurements of dopamine release.

Voltammetric measurements and analysis. For dopamine detection by fast-scan cyclic voltammetry, chronically implanted carbon-fiber microsensors were connected to a head-mounted voltammetric amplifier, interfaced with a PC-driven data-acquisition and analysis system (National Instruments) through an electrical swivel (Med Associates), which was mounted above the test chamber. Voltammetric scans were repeated every 100 ms to achieve a sampling rate of 10 Hz. During each voltammetric scan, the potential at the carbon-fiber electrode was linearly ramped from −0.4 V versus Ag/AgCl to +1.3 V (anodic sweep) and back (cathodic sweep) at 400 V/s (8.5 ms total scan time) and held at −0.4 V between scans. When dopamine is present at the surface of the electrode, it is oxidized during the anodic sweep to form dopamine-o-quinone (peak reaction detected at approximately +0.7 V), which is reduced back to dopamine in the cathodic sweep (peak reaction detected at approximately −0.3 V). The ensuing flux of electrons is measured as current and is directly proportional to the number of molecules that undergo electrolysis. The background-subtracted, time-resolved current obtained from each scan provided a chemical signature characteristic of the analyte, allowing resolution of dopamine from other substances (Phillips and Wightman, 2003). Dopamine was isolated from the voltammetric signal by chemometric analysis using a standard training set (Clark et al., 2010) based upon electrically stimulated dopamine release detected by chronically implanted electrodes. Dopamine concentration was estimated based upon the average postimplantation sensitivity of electrodes (Clark et al., 2010). Before analysis of average concentration, all data were smoothed with a 5-point within-trial running average. The concentration of dopamine during playback of acoustic stimuli (15 s) was averaged and compared with the average concentration 10 s before playback (baseline; Fig. 1*E*). Individual electrochemical signals were averaged across the first two presentations of each respective acoustic stimulus, and then across rats.

Behavioral analysis. Behavior during recording sessions was videotaped for analysis. The occurrence of orientation responses was assessed, including head orientations toward stimulus source and Preyer reflex (i.e., twitching of the ear). To measure locomotor activity, the operant box was divided into four quadrants (marked on the video monitor) and the numbers of transitions between quadrants were counted. Transitions were defined as interruptions of the quadrant borders by 50% of the rat's body (minus tail). To measure approach behavior toward the ultrasound source, an area with a radius of ~3.5 cm around the speaker was defined as proximal and time spent in this area was recorded. This time was defined as nose crossing into this area marked on the video monitor. Transitions and approach behavior were measured for a period of 10 s before (baseline), 15 s during, and 10 s after playback of acoustic stimuli. Values are presented as differences from baseline (baseline subtraction).

USV analysis. Playback of acoustic stimuli and USVs emitted by the subject rat were monitored using an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics). The microphone was connected via an UltraSoundGate 416H audio device (Avisoft Bioacoustics) to a PC, where acoustic data were recorded with a sampling rate of 250 kHz in 16-bit format by Avisoft RECORDER USGH. For acoustical analysis, recordings were transferred to Avisoft SASLab Pro and a fast Fourier transform (FFT) was conducted (512 FFT length, 100% frame, Hamming window, and 75% time window overlap), resulting in high-resolution spectrograms with a frequency range of 0–125 kHz (frequency resolution: 488 Hz; time resolution: 0.512 ms). An experienced user counted the number of 50 kHz USVs emitted by the subject rat by visual inspection of the spectrogram. Identification of 50 kHz USVs emitted by the subject rat was obtained by matching the playback stimulus with the recording acquired during playback (Wöhr and Schwarting, 2007). For all acoustic stimuli, the numbers of 50 kHz USVs emitted during the first two respective presentations were averaged, and then across rats. Values are presented as differences from baseline (baseline subtraction). No rat emitted 22 kHz USVs.

Histological verification of recording sites. Upon completion of experimentation, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Recording sites were marked with an electrolytic lesion (300 V) before transcardial perfusion with saline followed by 4% paraformaldehyde (PFA). Brains were removed and postfixed in PFA for 24 h and then rapidly frozen in an isopentane bath, sliced on a cryostat (50 μm coronal sections, −20°C), and stained with cresyl violet to aid in visualization of anatomical structures and the electrode-induced lesion.

Statistical analysis. Electrochemical signals and behavioral measures were compared using paired and unpaired *t* tests, as well as one-way and two-way ANOVAs for repeated measurements (acoustic stimulus; time). When appropriate, one-tailed *post hoc* analyses were conducted, and *p* values were adjusted according to the Holm–Bonferroni correction method for multiple testing (Wright, 1992). Statistical analyses were performed using SPSS (version 17.0, IL) and Prism (GraphPad Software). Graphical representations were generated using Prism. A p value of <0.05 was considered statistically significant.

Results

Behavioral changes in response to playback of acoustic stimuli

All four types of acoustic stimuli elicited orientation responses, with response rates being similarly high and gradually decreasing with repeated exposures, regardless of stimulus (overall orientation response rate: ~60%; main effect acoustic stimulus: $F_{(3,33)} = 1.184$, $p = 0.331$).

Playback of 50 kHz USVs increased locomotor activity and led to approach behavior in the recipient rat, with behavioral responses being most prominent during the first few presentations. Locomotor activity, as measured by means of quadrant line crossings, was significantly increased in response to the first four 50 kHz USV presentations ($t_{(11)} = 2.933$, $p = 0.014$, $t_{(11)} = 2.258$, $p = 0.045$, $t_{(11)} = 3.530$, $p = 0.005$, and $t_{(11)} = 2.866$, $p = 0.015$, respectively; all other $p > 0.050$; Figure 2*A*). Significant approach behavior, as assessed by the time spent in proximity to the ultrasonic speaker, was seen in response to the first two 50 kHz USV presentations ($t_{(11)} = 3.644$, $p = 0.004$ and $t_{(11)} = 3.191$, $p = 0.009$, respectively; all other $p > 0.050$; Figure 2*B*). The behavioral significance of this measure dictated subsequent analyses concentrating on the first two stimulus presentations. When focusing the analysis on the first two presentations, clear behavioral responses were observed specifically in response to playback of 50 kHz USVs, with playback of acoustic stimuli affecting locomotor activity and approach behavior in a stimulus-specific manner (main effect acoustic stimulus; $F_{(3,33)} = 4.325$, $p = 0.011$ and $F_{(3,33)} = 3.587$, $p = 0.024$, respectively; Figure 2*C*,*D*): 50 kHz USVs significantly increased locomotor activity ($t_{(11)} = 3.876$, $p = 0.003$), whereas such a response was not seen during playback of 22 kHz USVs ($t_{(11)} = 1.386$, $p = 0.193$), NOISE ($t_{(11)} = 1.700$, $p = 0.117$), or BACKGROUND ($t_{(11)} = 1.744$, $p = 0.109$; Fig. 2*C*). Locomotor activity during 50 kHz USV playback thus exceeded changes observed in response to playback of 22 kHz USVs, NOISE, or BACKGROUND (all $p < 0.050$; Fig. 2*C*). Similar results were obtained for approach behavior. During the first two playback sessions, the time spent in proximity to the ultrasonic speaker significantly increased during 50 kHz USV playback ($t_{(11)} = 4.102$, $p = 0.002$), while such a response was less prominent or

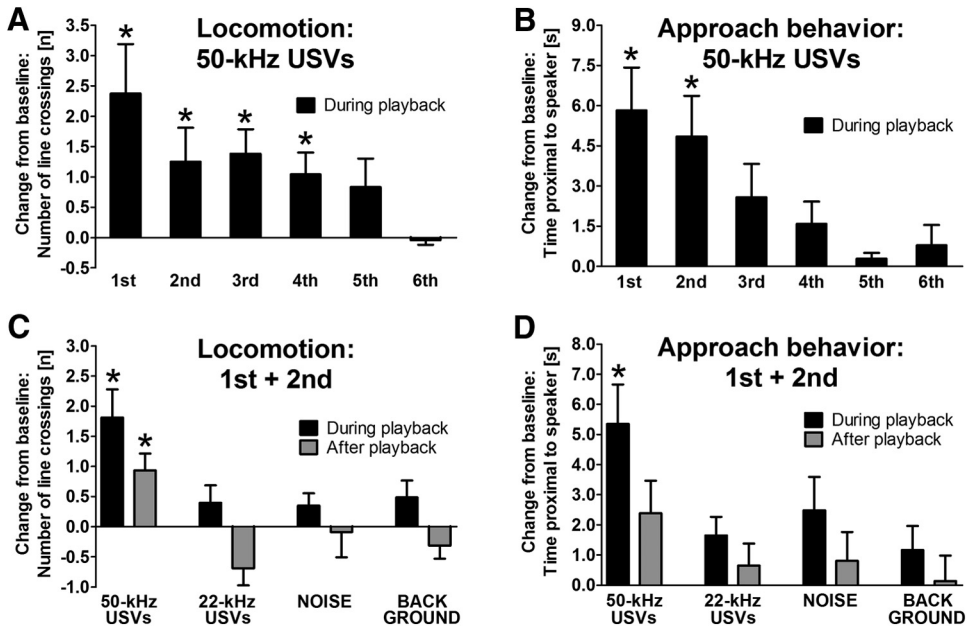

Figure 2. Behavioral responses to playback of acoustic stimuli. ***A***, Change from baseline in locomotion measured by counting the number of quadrant line crossings, and ***B***, Change from baseline in the time spent proximal to the ultrasonic loudspeaker in response to the first six presentations of 50 kHz USVs on the first day of the experiment; $*p < 0.050$, 50 kHz USVs compared with baseline. ***C***, Change in locomotion, and ***D***, change in the time spent proximal to the ultrasonic loudspeaker in response to the first two presentations of 50 and 22 kHz USVs, time- and amplitude-matched white noise (NOISE), and background noise (BACKGROUND) on the first day of the experiment; $*p < 0.050$, 50 kHz USVs compared with all other acoustic stimuli. Data are presented as differences from baseline (baseline subtraction); mean + SEM ($n = 12$ rats for each presentation).

absent in response to playback of 22 kHz USVs ($t_{(11)} = 2.660$, $p = 0.022$), NOISE ($t_{(11)} = 2.252$, $p = 0.046$), or BACKGROUND ($t_{(11)} = 1.453$, $p = 0.174$; Fig. 2*D*), resulting in higher levels of approach behavior during playback of 50 kHz USVs, as compared with the three other stimuli (all $p < 0.050$; Fig. 2*D*).

Similarly to the playback phase, locomotor activity displayed after playback was also affected in a stimulus-specific manner, indicating that playback-induced behavioral changes outlasted the termination of playback (main effect acoustic stimulus; $F_{(3,33)} = 4.813$, $p = 0.007$; Fig. 2*C*): locomotor activity remained higher following playback of 50 kHz USVs than after 22 kHz USVs, NOISE, or BACKGROUND (all $p < 0.05$; Fig. 2*C*), of which the 22 kHz USVs even led to behavioral inhibition ($t_{(11)} = -2.421$, $p = 0.034$; Fig. 2*C*). In contrast, although the 50 kHz USV-induced increase in approach behavior immediately following the termination of playback was greater than after playback of the other sounds, this difference was not significant (main effect acoustic stimulus; $F_{(3,33)} = 1.049$, $p = 0.384$; Fig. 2*D*).

Finally, a substantial increase in the emission rates of 50 kHz USVs (more than one call per second) was detected in three recipient rats in response to the first playback of 50 kHz USVs, with the remaining rats displaying only moderate or no increase in 50 kHz USVs. Overall, emission of 50 kHz USVs tended to increase during and after the first 50 kHz USV presentation ($t_{(10)} = 1.866$, $p = 0.092$ and $t_{(10)} = 2.023$, $p = 0.071$; all other $p > 0.100$), whereas no substantial increase in 50 kHz USV emission was evident in response to the other acoustic stimuli presented (all $p > 0.100$). When focusing the analysis on the first two presentations in which behavioral responses were most prominent, more 50 kHz USVs tended to be emitted in response to playback of 50 kHz USVs than when exposed to 22 kHz USVs, NOISE, and BACKGROUND, during and after playback (main effect acoustic stimulus; $F_{(3,30)} = 2.354$, $p = 0.092$ and $F_{(3,30)} = 2.707$, $p = 0.063$, respectively; data not shown).

Together, these results suggest that the increase in locomotor activity observed during playback of 50 kHz USVs was directed toward the sound source, indicating social approach behavior, occasionally paralleled by a 50 kHz USV emission. No such behavioral response pattern was seen during the exposure to the three other acoustic stimuli.

Dopamine release in response to playback of acoustic stimuli

To investigate the relationship between USV-induced social behavior and dopamine release in the NAcc, we measured electrochemical changes in this brain region using fast-scan cyclic voltammetry in awake, behaving rats. Histological analyses demonstrated that average dopamine release in the NAcc core (6.262 ± 2.586 nM) and shell (5.980 ± 3.746 nM) did not differ significantly in response to the 50 kHz USV presentation ($t_{(14)} = 3.121$, $p = 0.951$). Therefore, data from these two subregions were pooled for the following analyses.

Paralleling the behavioral effects, changes in phasic dopamine release were seen during playback of acoustic stimuli, with the observed effects again being stimulus-specific. Specifically, during 50 kHz USV playback, we observed a transient increase in extracellular dopamine concentration over baseline ($t_{(14)} = 3.121$, $p = 0.008$; Figs. 3*A*,*C*, 4*A*). In contrast, no increase was observed during playback of 22 kHz USVs ($t_{(14)} = 1.110$, $p = 0.286$; Figs. 3*B*,*D*, 4*B*), NOISE ($t_{(14)} = 1.440$, $p = 0.172$; Fig. 4*C*), or BACKGROUND ($t_{(14)} = 0.102$, $p = 0.920$; Fig. 4*D*). Thus, changes in dopamine neurotransmission were dependent on the type of acoustic stimulus used for playback (main effect acoustic stimulus; $F_{(3,59)} = 6.224$, $p = 0.001$; Fig. 4*E*), with the increase in dopamine release elicited by 50 kHz USV presentation being significantly greater than during playback of 22 kHz USVs, NOISE, or BACKGROUND (all $p < 0.010$; Fig. 4*E*). Importantly, the increase in dopamine release during 50 kHz USV playback was not significantly correlated with locomotor activity per se ($r =$

0.183, $p = 0.569$), but specifically with approach behavior toward the sound source ($r = 0.610$, $p = 0.035$; Fig. 4*F*).

To further characterize the signal, we compared the average peak increase of the dopamine response to 50 kHz USV playback (23.3 ± 1.9 nM) to the response to the unexpected delivery of a sucrose pellet (26.8 ± 2.1 nM): the responses did not differ significantly in magnitude ($t_{(22)} = 1.218$, $p = 0.236$; data not shown). In a separate analysis, we divided the animals into two populations based on whether they responded to 50 kHz USV playback with the production of 50 kHz USVs. However, our results show that the 50 kHz USV effect on dopamine release was not different between low- and high-vocalizing rats ($t_{(12)} = 0.918$, $p = 0.377$; data not shown), indicating that dopamine release consistently occurs during exposure to 50 kHz USVs regardless of whether the subject rats emitted 50 kHz USVs in response. In line with that, increases in 50 kHz USV emission and dopamine release were not significantly correlated ($r = 0.110$, $p = 0.747$).

Paralleling behavioral changes, effects of 50 kHz USVs on dopamine signaling were dependent on the number of presentations (main effect time; $F_{(2,28)} = 9.837$, $p = 0.001$; Fig. 4*G*). Specifically, the effects on dopamine signaling were most pronounced during the first two playback exposures, but then declined rapidly with subsequent presentations (all $p < 0.010$; Fig. 4*G*). Moreover, on the second day of the experiment, playback of acoustic stimuli was without effect on locomotor activity (main effect acoustic stimulus; $F_{(3,30)} = 0.957$, p=0.426), approach behavior (main effect acoustic stimulus; $F_{(3,30)} = 2.384$, $p = 0.089$), and dopamine (main effect acoustic stimulus; $F_{(3,36)} = 2.070$, $p = 0.121$). Consistently, none of the acoustic stimuli produced a significant neurochemical change from baseline during presentation on the second day of the experiment (trace for 50 kHz USVs in Fig. 4*H*; other stimuli not shown; $t_{(12)} = 0.727–2.005$, $p = 0.272–0.481$).

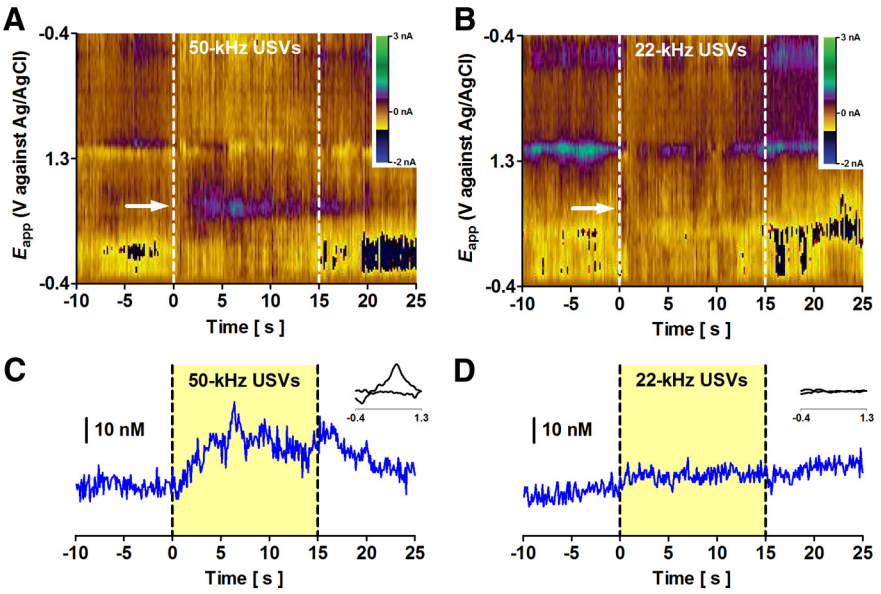

Figure 3. Changes in dopamine release in the NAcc in response to playback of acoustic stimuli. ***A***, Exemplary pseudocolor plot of the change in phasic dopamine release associated with a single playback presentation of 50 kHz USVs. ***B***, Exemplary pseudocolor plot of the change in phasic dopamine release associated with a single playback presentation of 22 kHz USVs. ***C***, Change in dopamine concentration and cyclic voltammograms (inset) for representative current fluctuations shown in ***A*** for the period 10 s before the beginning of the 50 kHz USV presentation (left dashed line), during the 15 s presentation (yellow box), and 10 s after the offset (right dashed line). ***D***, Change in dopamine concentration and cyclic voltammograms (inset) for representative current fluctuations shown in ***B*** for the period 10 s before the beginning of the 22 kHz USV presentation (left dashed line), during the 15 s presentation (yellow box), and 10 s after the offset (right dashed line).

Discussion

Under natural conditions, rats emit 50 kHz USVs mainly during appetitive social interactions, such as rough-and-tumble play in juveniles (Knutson et al., 1998) or mating in adulthood (Sales, 1972). Panksepp et al. (2002) further found that rats spend more time with conspecifics emitting high levels of 50 kHz USVs than with others producing fewer 50 kHz USVs and that deafening rats affects rough-and-tumble-play (Siviy and Panksepp, 1987). Similarly, findings of Brudzynski and Pniak (2002) suggest that the 50 kHz USV emission is driven by prospective social contact. Remarkably, 50 kHz USVs were also reported to correlate with social cooperative behavior (Łopuch and Popik, 2011). This indicates that 50 kHz USVs serve a pro-social communicative function as contact calls, which are important for maintaining social proximity and coordinating behavior in groups of rats—a view that was recently supported by means of a series of playback experiments showing that 50 kHz USVs induce social approach behavior in the recipients (Wöhr and Schwarting, 2007, 2009, 2012).

However, little is known about the neurobiology that underlies socio-affective ultrasonic communication in rats. Therefore, we made use of our established 50 kHz USV playback paradigm (Wöhr and Schwarting, 2007, 2009, 2012) and combined it with fast-scan cyclic voltammetry to measure phasic dopamine release during USV playback in freely moving rats (Clark et al., 2010; Flagel et al., 2011; Willuhn et al., 2012). We demonstrate that 50 kHz USVs can elicit phasic dopamine release in the NAcc, along with social exploratory behavior directed toward the sound source, consistent with previous behavioral studies (Wöhr and Schwarting, 2007, 2009, 2012) and in agreement with a pro-social communicative function of 50 kHz USVs (Siviy and Panksepp, 1987; Brudzynski and Pniak, 2002; Panksepp et al., 2002).

Our finding is also consistent with results from a study by Robinson et al. (2002) showing that the frequency of phasic dopamine release events, so-called transients, increases in the presence of a conspecific rat. By means of our tightly controlled experimental conditions under which olfactory, visual, and tactile cues informing about the presence of a conspecific rat were removed, we detected an increase in NAcc dopamine release that is time locked to the presentation of 50 kHz USVs. Thus, we identified a communicative signal that may in fact be responsible for the increase in the frequency of dopamine transients reported by Robinson et al. (2002).

Importantly, we demonstrate that simply not every acoustic stimulus that indicates the presence of another conspecific rat elicits NAcc dopamine release, as the presentation of 22 kHz USVs, which typically occur in aversive situations such as predator exposure (Blanchard et al., 1991), fear conditioning (Wöhr and Schwarting, 2008), or social defeat (Kroes et al., 2007), had no effect on dopamine release but evoked behavioral inhibition, in line with their presumed alarming function (Blanchard et al., 1991; Endres et al., 2007; Wöhr and Schwarting, 2007, 2008).

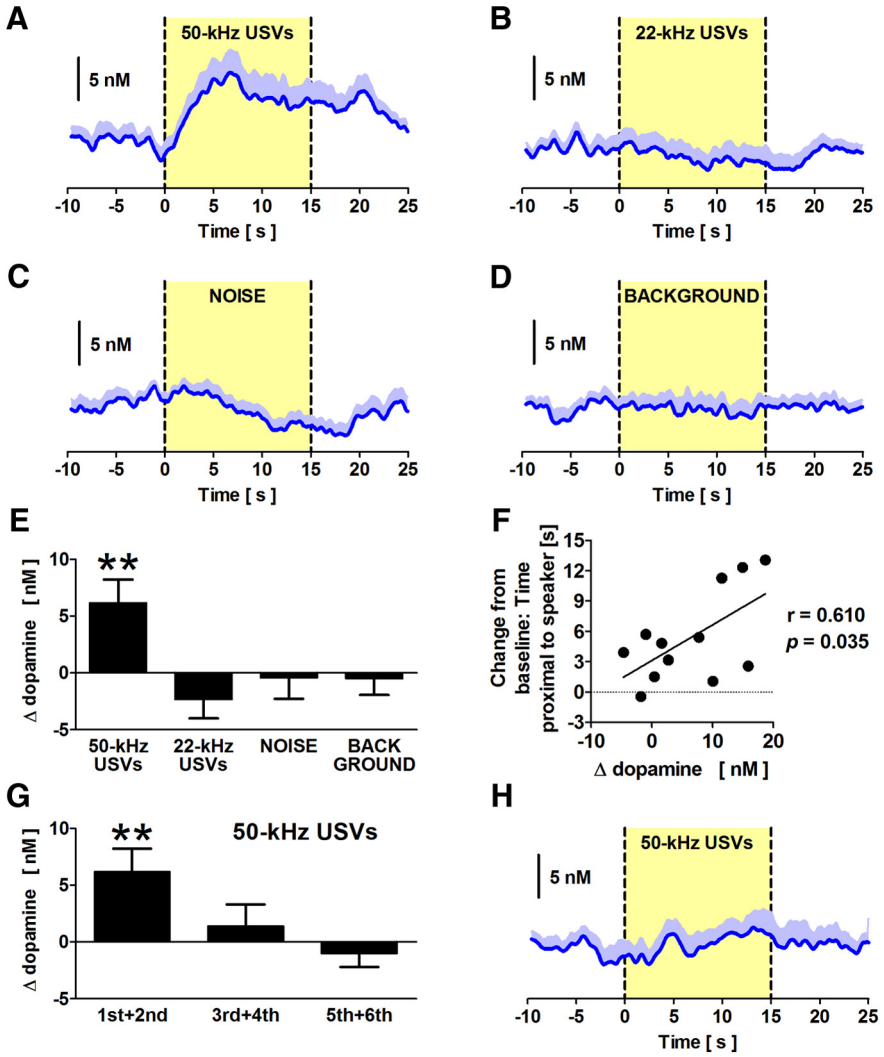

Figure 4. Average changes in dopamine release in the NAcc in response to playback of acoustic stimuli, namely the first two presentations of (***A***) 50 kHz USVs, (***B***) 22 kHz-USVs, (***C***) time- and amplitude-matched white noise (NOISE), and (***D***) background noise (BACKGROUND) on the first day of the experiment. ***E***, The increase in dopamine release during the 50 kHz USV playback is significantly greater than during the other acoustic stimuli. ***F***, The increase in dopamine release during 50 kHz USV playback is significantly correlated with approach behavior toward the sound source. ***G***, The increase in dopamine release during playback of the first two 50 kHz USVs is significantly greater than subsequent presentations. ***H***, Average change in dopamine release in the NAcc in response to playback of acoustic stimuli, namely the first two presentations of 50 kHz USVs on the second day of the experiment ($n = 10$); $^{**}p < 0.010$. Data are presented as differences from baseline (baseline subtraction); mean + SEM ($n = 12$ rats for each presentation unless indicated otherwise).

Furthermore, the sound of a rat moving on cage bedding, i.e., the background noise stimulus, also predictive of the presence of a conspecific rat, had no effect on NAcc dopamine release either. Thus, phasic dopamine release in the NAcc and behavioral activation were exclusively seen in response to playback of 50 kHz USVs, indicating that not merely the anticipation or the presence of a conspecific rat is eliciting NAcc dopamine release, but the actual communicative signal emitted by conspecific rats.

We further assured specificity of this effect by demonstrating that playback of time- and amplitude-matched white noise did not evoke phasic dopamine release. For this purpose, time- and amplitude-matched white noise was generated by replacing 50 kHz USVs with white noise, where duration and amplitude modulation corresponded to that of the 50 kHz USVs. Thus, white noise was identical to the original natural 50 kHz USV series with respect to all call features including temporal pattern, but with the exception that sound energy was not confined to a certain frequency. In this context, it is important to highlight that all four acoustic stimuli presented elicited orientation responses in a similar number, indicating that all of them were audible and that differences in dopamine release do not result from differences in their potency to evoke attention. Finally, we performed analyses that show that the correlation between dopamine and locomotion (a motor component of approach) was not significant, whereas the correlation between dopamine and approach was. Thus, the increase in dopamine release during 50 kHz USV playback was not attributable to locomotor activity per se, but was specifically associated with approach behavior toward the sound source. Together, this shows that the phasic dopamine response to playback of 50 kHz USV recordings was not due to movement, novelty, or general acoustic features also present in the control stimuli tested, but instead required the perception of unique acoustic features specifically linked to the communicative function of 50 kHz USVs.

The observed effects on both neurochemistry and behavior were most pronounced during initial playback, but then declined rapidly with subsequent presentations, pointing at a close relationship between the two measures. This rapid decline during 50 kHz USV exposure is consistent with a previous study (Wöhr and Schwarting, 2012), showing that high levels of social approach behavior occur in response to 50 kHz USVs when rats are exposed to them for the first time, but not in response to subsequent exposures. The decline in social approach behavior during repeated exposures to 50 kHz USVs is probably due to memory processes, since it can be blocked by post-trial administration of the amnesia-inducing drug scopolamine (Wöhr and Schwarting, 2012). In contrast, the dopamine-releasing effects of unexpected delivery of consumable rewards such as sucrose pellets or its respective prediction are stable across many presentations (Day et al., 2007; Flagel et al., 2011). However, the dopamine response during initial playback of 50 kHz USVs was comparable in amplitude to dopamine release induced by the unexpected delivery of food rewards. Together, these findings indicate that 50 kHz USVs transiently exhibit the same incentive function and involve the same neural systems as a consumable reward, but then dissipate this property much faster than food reward, suggesting that these pro-social calls are not perceived as primary rewards and lose their value quickly when not reinforced.

The finding that phasic dopamine release in NAcc encodes pro-social 50 kHz USVs, but not alarming 22 kHz USVs, supports the notion that the two USV types are processed by distinct brain

areas, involved in regulating approach and avoidance, respectively. Alarming 22 kHz USVs were found to induce activation in amygdala and periaqueductal gray (Sadananda et al., 2008; Parsana et al., 2012), brain areas strongly implicated in the regulation of anxiety and fear (Fendt and Fanselow, 1999; LeDoux, 2000), whereas 50 kHz USVs inhibit the amygdala (Parsana et al., 2012), but evoke neuronal activation in the NAcc, as shown by means of c-*fos* immunohistochemistry (Sadananda et al., 2008), in line with our present findings.

Stimulating dopamine neurotransmission by means of amphetamine or cocaine is known to result in 50 kHz USV emission (Barker et al., 2010; Ahrens et al., 2013; Wright et al., 2013; Pereira et al., 2014), which can be blocked by dopamine antagonists (Burgdorf et al., 2001; Thompson et al., 2006; Wright et al., 2013). Furthermore, and particularly relevant in the present context, microinfusion of amphetamine into the NAcc, particularly shell, but also core, elicits a strong increase in 50 kHz USV production, while not eliciting 22 kHz USVs (Burgdorf et al., 2001; Thompson et al., 2006), whereas electrolytic and 6-OHDA lesions of the ventral tegmental area selectively reduce 50 kHz USVs, but not 22 kHz USVs (Burgdorf et al., 2007). Here we now show for the first time that NAcc dopamine release is not just linked to the production of 50 kHz USVs, but also the perception of 50 kHz USVs, possibly indicating that the NAcc functions to close a perception-and-action-loop, which is particularly relevant for appetitive social and reciprocal communicatory signals. However, our present findings show that NAcc dopamine release in response to 50 kHz USV playback is paralleled by the production of 50 kHz USVs in only a subset of the tested animals (and no increase in calling in response to the other stimuli). In other words, 50 kHz USV production only occurred after phasic dopamine release, but dopamine release was not always followed by call production. This suggests that NAcc dopamine release following the perception of 50 kHz USVs may be necessary, but not sufficient, for the production of 50 kHz USVs.

In contrast, we found that dopamine release was positively correlated with approach behavior, indicating a relationship between these two variables present in the entire population of the tested animals. Based on many previous studies that demonstrate an invigorating function of dopamine release (Cardinal et al., 2002; Salamone et al., 2007; Nicola, 2010), we speculate that dopamine transmission in response to 50 kHz USVs promoted appetitive approach, rather than being a product of appetitive approach. In support of this interpretation, Flagel et al. (2011) found that during Pavlovian conditioning rats approached either the predictive cue or the location of the predicted reward. Rats that approached the reward-predictive cue displayed NAcc dopamine release in response to cue presentation, whereas rats that approached the reward location did not. Thus, in both cases the animals showed approach behavior, but only in one case dopamine signaling was critical. Therefore, dopamine release and approach behavior are dissociable, indicating that dopamine release is not induced by approach behavior per se. Thus, our present findings suggest that NAcc dopamine functions as a translator of a motivational acoustic signal into a pro-social action, thereby establishing a functional link between the perception of appetitive communicative signals and reward-related neurotransmission.

References

Ahrens AM, Nobile CW, Page LE, Maier EY, Duvauchelle CL, Schallert T (2013) Individual differences in the conditioned and unconditioned rat 50-kHz ultrasonic vocalizations elicited by repeated amphetamine exposure. Psychopharmacology 229:687–700. CrossRef Medline

Barker DJ, Root DH, Ma S, Jha S, Megehee L, Pawlak AP, West MO (2010) Dose-dependent differences in short ultrasonic vocalizations emitted by rats during cocaine self-administration. Psychopharmacology 211:435–442. CrossRef Medline

Blanchard RJ, Blanchard DC, Agullana R, Weiss SM (1991) Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems. Physiol Behav 50:967–972. CrossRef Medline

Brudzynski SM (2013) Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. Curr Opin Neurobiol 23:310–317. CrossRef Medline

Brudzynski SM, Pniak A (2002) Social contacts and production of 50-kHz short ultrasonic calls in adult rats. J Comp Psychol 116:73–82. CrossRef Medline

Burgdorf J, Knutson B, Panksepp J, Ikemoto S (2001) Nucleus accumbens amphetamine microinjections unconditionally elicit 50-kHz ultrasonic vocalizations in rats. Behav Neurosci 115:940–944. CrossRef Medline

Burgdorf J, Wood PL, Kroes RA, Moskal JR, Panksepp J (2007) Neurobiology of 50-kHz ultrasonic vocalizations in rats: electrode mapping, lesion, and pharmacology studies. Behav Brain Res 182:274–283. CrossRef Medline

Burman OHP, Ilyat A, Jones G, Mendl M (2007) Ultrasonic vocalizations as indicators of welfare for laboratory rats (Rattus norvegicus). Appl Anim Behav Sci 104:116–129. CrossRef

Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002) Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. Neurosci Biobehav Rev 26:321–352. CrossRef Medline

Clark JJ, Sandberg SG, Wanat MJ, Gan JO, Horne EA, Hart AS, Akers CA, Parker JG, Willuhn I, Martinez V, Evans SB, Stella N, Phillips PE (2010) Chronic microsensors for longitudinal, subsecond dopamine detection in behaving animals. Nat Methods 7:126–129. CrossRef Medline

Day JJ, Roitman MF, Wightman RM, Carelli RM (2007) Associative learning mediates dynamic shifts in dopamine signaling in the nucleus accumbens. Nat Neurosci 10:1020–1028. CrossRef Medline

Endres T, Widmann K, Fendt M (2007) Are rats predisposed to learn 22 kHz calls as danger-predicting signals? Behav Brain Res 185:69–75. CrossRef Medline

Fendt M, Fanselow MS (1999) The neuroanatomical and neurochemical basis of conditioned fear. Neurosci Biobehav Rev 23:743–760. CrossRef Medline

Flagel SB, Clark JJ, Robinson TE, Mayo L, Czuj A, Willuhn I, Akers CA, Clinton SM, Phillips PE, Akil H (2011) A selective role for dopamine in stimulus-reward learning. Nature 469:53–57. CrossRef Medline

Knutson B, Burgdorf J, Panksepp J (1998) Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. J Comp Psychol 112:65–73. CrossRef Medline

Kroes RA, Burgdorf J, Otto NJ, Panksepp J, Moskal JR (2007) Social defeat, a paradigm of depression in rats that elicits 22-kHz vocalizations, preferentially activates the cholinergic signaling pathway in the periaqueductal gray. Behav Brain Res 182:290–300. CrossRef Medline

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23:155–184. CrossRef Medline

Łopuch S, Popik P (2011) Cooperative behavior of laboratory rats (Rattus norvegicus) in an instrumental task. J Comp Psychol 125:250–253. CrossRef Medline

Mogenson GJ, Jones DL, Yim CY (1980) From motivation to action: functional interface between the limbic system and the motor system. Prog Neurobiol 14:69–97. CrossRef Medline

Nicola SM (2010) The flexible approach hypothesis: unification of effort and cue-responding hypotheses for the role of nucleus accumbens dopamine in the activation of reward-seeking behavior. J Neurosci 30:16585–16600. CrossRef Medline

Panksepp J, Gordon N, Burgdorf J (2002) Empathy and the action-perception resonances of basic socio-emotional systems of the brain. Behav Brain Sci 25:43–44.

Parsana AJ, Li N, Brown TH (2012) Positive and negative ultrasonic social signals elicit opposing firing patterns in rat amygdala. Behav Brain Res 226:77–86. CrossRef Medline

Pereira M, Andreatini R, Schwarting RKW, Brenes JC (2014) Amphetamine-induced appetitive 50-kHz calls in rats: a marker of affect in mania? Psychopharmacology 2231:2567–2577. CrossRef

Phillips PE, Wightman RM (2003) Critical guidelines for validation of the selectivity of in-vivo chemical microsensors. Trends Analyt Chem 22: 509–514. CrossRef

Portfors CV (2007) Types and functions of ultrasonic vocalizations in laboratory rats and mice. J Am Assoc Lab Anim Sci 46:28–34. Medline

Robbins TW, Everitt BJ (1982) Functional studies of the central catecholamines. Int Rev Neurobiol 23:303–365. CrossRef Medline

Robinson DL, Heien ML, Wightman RM (2002) Frequency of dopamine concentration transients increases in dorsal and ventral striatum of male rats during introduction of conspecifics. J Neurosci 22:10477–10486. Medline

Sadananda M, Wöhr M, Schwarting RK (2008) Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. Neurosci Lett 435:17–23. CrossRef Medline

Salamone JD, Cousins MS, Bucher S (1994) Anhedonia or anergia? Effects of haloperidol and nucleus accumbens dopamine depletion on instrumental response selection in a T-maze cost/benefit procedure. Behav Brain Res 65:221–229. CrossRef Medline

Salamone JD, Correa M, Farrar A, Mingote SM (2007) Effort-related functions of nucleus accumbens dopamine and associated forebrain circuits. Psychopharmacology 191:461–482. CrossRef Medline

Sales GD (1972) Ultrasound and mating behaviour in rodents with some observations on other behavioural situations. J Zool 168:149–164.

Schultz W, Dayan P, Montague PR (1997) A neural substrate of prediction and reward. Science 275:1593–1599. CrossRef Medline

Siviy SM, Panksepp J (1987) Sensory modulation of juvenile play in rats. Dev Psychobiol 20:39–55. CrossRef Medline

Thompson B, Leonard KC, Brudzynski SM (2006) Amphetamine-induced 50 kHz calls from rat nucleus accumbens: a quantitative mapping study and acoustic analysis. Behav Brain Res 168:64–73. CrossRef Medline

Vivian JA, Barros HM, Manitiu A, Miczek KA (1997) Ultrasonic vocalizations in rat pups: modulation at the gamma-aminobutyric acid A receptor complex and the neurosteroid recognition site. J Pharmacol Exp Ther 282:318–325. Medline

Willuhn I, Burgeno LM, Everitt BJ, Phillips PE (2012) Hierarchical recruitment of phasic dopamine signaling in the striatum during the progression of cocaine use. Proc Natl Acad Sci U S A 109:20703–20708. CrossRef Medline

Wöhr M, Schwarting RK (2007) Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? PLoS One 2:e1365. CrossRef Medline

Wöhr M, Schwarting RK (2009) Ultrasonic communication in rats: effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations. Pharmacol Biochem Behav 94:285–295. CrossRef Medline

Wöhr M, Schwarting RK (2012) Testing social acoustic memory in rats: effects of stimulus configuration and long-term memory on the induction of social approach behavior by appetitive 50-kHz ultrasonic vocalizations. Neurobiol Learn Mem 98:154–164. CrossRef Medline

Wöhr M, Schwarting RK (2013) Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. Cell Tissue Res 354:81–97. CrossRef Medline

Wöhr M, Schwarting RKW (2008) Ultrasonic calling during fear conditioning in the rat: no evidence for an audience effect. Anim Behav 76:749–760. CrossRef

Wright JM, Dobosiewicz MR, Clarke PB (2013) The role of dopaminergic transmission through D1-like and D2-like receptors in amphetamine-induced rat ultrasonic vocalizations. Psychopharmacology 225:853–868. CrossRef Medline

Wright SP (1992) Adjusted *p*-values for simultaneous inference. Biometrics 48:1005–1013. CrossRef