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Research report

Neurobiology of 50-kHz ultrasonic vocalizations in rats: Electrode mapping, lesion, and pharmacology studies

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Abstract

Fifty-kHz ultrasonic vocalizations have been proposed to reflect a positive appetitive affective state in rats, being consistently linked to the positive appetitive behavior. In the first study, we examined the brain substrates of 50-kHz ultrasonic vocalizations (USVs) by using localized electrical stimulation of the brain (ESB) at various sites that are known to mediate reward. We found that the brain areas that produced ESB-induced 50-kHz calls are the areas that have previously been shown to support the most vigorous self-stimulation behavior (prefrontal cortex, nucleus accumbens, ventral pallidum, lateral preoptic area, lateral hypothalamus, ventral tegmental area, and raphe). Importantly, all animals that showed repeatable ESB-induced 50-kHz USVs demonstrated self-stimulation behavior. In the second study, conditioned place preference was assessed following microinjection of the μ-opiate agonist Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol (DAMGO) directly into the ventral tegmental area (VTA) at a dose previously found to be rewarding. Animals that showed more 50-kHz USVs in response to drug injections compared to vehicle injections showed significant place preferences, whereas animals that did not show elevated vocalization to DAMGO did not show place preference. In experiment 3, we examined the effect of VTA electrolytic lesions, 6-OHDA lesions, and the effect of the D1/D2 dopamine antagonist flupenthixol (0 and 0.8 mg/kg, i.p.) on 50-kHz ultrasonic vocalizations. We found that these manipulations all selectively reduced 50-kHz ultrasonic vocalizations, and that these effects could be disassociated from any side effects. These data are consistent with the proposition that 50-kHz calls are tightly linked to reward in rats and that the neural circuit of 50-kHz calls closely overlaps that of ESB self-stimulation reward, drug reward, and the mesolimbic dopamine system.

Keywords: Ultrasonic vocalizations; Rats; Emotion; Electrical stimulation of the brain; Reward; DAMGO; Dopamine

1. Introduction

Our laboratory has shown that rat 50-kHz ultrasonic vocalizations (USVs) may index a positive affective state associated with rewarding brain states [17]. Previously, we have shown that 50-kHz USVs are elevated by food reward, sexual behavior, rough-and-tumble play, drugs of abuse, and anticipation of rewarding electrical brain stimulation [2–4,15,16]. Conversely, aversive stimuli including bright light, predatory odors, foot shock, and drugs with aversive properties *decrease* levels of 50-kHz USVs [4,16], while increasing rates of 22-kHz USVs [7,19]. During heterospecific play behavior (i.e. "tickling", which mimics rough-and-tumble play), rates of 50-kHz USVs are consistently positively correlated with the rewarding value of the stimulation as measured by instrumental approach behavior across several studies [5,25].

The neurobiology of reward has classically been studied by examining brain areas that support self-administration of electrical brain stimulation (self-stimulation) or by examining the rewarding effects of drugs injected directly into the brain and studying their ability to support self administration, facilitate electrical self-stimulation, or produce conditioned place preferences [11,33]. While a number of brain areas electrical self-stimulation behavior, the circuits from the prefrontal cortex through the medial forebrain bundle to the raphe are associated with the most robust reward, at least as measured by rates

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of responding [9,22]. Within this circuit, the ventral tegmental area (VTA) has been the most consistent brain region across studies that support the rewarding effects of opiates, nicotine, barbiturates, and neurotensin when given focally [12].

A strong case has been made that 50-kHz USVs in rats index a state of positive affect that accompanies many kinds of appetitive rewards [6,17,25]. To the extent that the mesolimbic self-stimulation trajectory of dopamine mediated euphoric arousal reflects a generalized *seeking* urge that is necessary for acquisition of all specific rewards [24], it would be expected that 50-kHz USVs would be strongly regulated by this appetitive system of the brain. Accordingly, the aim of the following work was to evaluate the degree to which the mesolimbic dopamine system participates in the regulation of this vocal indicator of reward and positive affect.

The purpose of the present studies was to test the hypothesis that animals that receive either electrical stimulation or chemical stimulation to the brain that increases 50-kHz USVs will also find stimulation to be rewarding as measured by electrical self-stimulation (experiment 1) or by conditioned place preference in drug microinjection studies (experiment 2). In addition, we tested the hypothesis that disruptions in the mesolimbic dopamine system, which has been shown to play an important role in reward and 50-kHz USVs will decrease rates of 50-kHz USVs. Experiment 3 evaluated that proposition through the use of electrolytic and 6-OHDA lesions of the VTA region as well as changes in 50-kHz USVs following dopamine receptor blockade. It was anticipated that all manipulations reducing brain dopamine activity would reduce the emission of 50-kHz USVs.

2. Methods

Female Long Evans rats born and bred in the Bowling Green State University animal facilities were used in this study. Females were used across all of the following studies given that they show higher rates of 50-kHz USVs in adulthood in response to "tickling". All animals were weaned at 21 days of age and singly housed in 20 cm × 40 cm × 20 cm Lucite cages with corn-cob bedding. Subjects were maintained on a 12:12 light dark cycle (lights on 8 a.m.) tested during the light phase, and were given *ad libitum* access to Purina lab chow and tap water throughout the study.

2.1. Surgery experiment 1

Subjects were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and mounted in a Kopf stereotactic instrument (Kopf Instruments, USA). After exposing the skull, bregma and lambda were visualized and aligned on the same horizontal plane. Bipolar stainless steel electrodes (Plastics One, USA) were lowered into the brain, and in a subset of animals, 23 gauge guide cannulae (Plastics One, USA) were aimed for the contralateral VTA (stereotaxic coordinates from bregma: −5.0 AP, 1.0 ML, 8.0 DV, flat brain). Cannulae and electrodes were angled 16° away from the midline to avoid ventricles. Four additional holes were drilled for insertion of stainless steel self-tapping anchor screws, and electrodes (with the distance between the active electrodes at tip set at ∼0.5 mm) were secured to the skull with dental acrylic.

2.2. Experiment 1: electrical brain stimulation testing

Forty-seven female Long Evans rats were used in this study. After a minimum of a week of post-surgical recovery, animals first received experimenter delivered brain stimulation to test for stimulation induced USVs using a protocol shown to elicit the maximal dopamine release from electrical stimulation of the VTA consisting, of three 10 s 120 μA stimulus trains of continuous 60 Hz sine wave stimulation (Lafayette Instrument Sine Wave Stimulator, Model 82408, USA), with a 30 min inter-train interval; see [8,23]. Current thresholds for stimulation-induced vocalizations, motor behaviors (e.g. turning) as well as stimulation induced feeding were conducted in a 45 cm × 35 cm × 20 cm opaque box with laboratory rat chow on the floor. Animals were given ascending 10 s continuous pulses of ESB starting at 5 μA, and increasing in 5 μA steps until a behavioral effect (i.e. stimulus bound feeding, vocalizations or turning) emerged with at least 10 s between stimulations. After the identification of an active site, the stimulation was repeated four times, and if the stimulation elicited the behavior in at least 3 of 4 trials, the current level was recorded as the threshold level.

For self-stimulation tests, subjects were placed in a 34 cm × 23 cm × 32.5 cm translucent box with a 10 cm × 2 cm × 25 cm bar elevated 8.5 cm from the floor. Subjects were allowed to bar press for 0.5–0.2 s trains of 50–200 μA pulses on a continuous reinforcement schedule, as in Ref. [27]. During this training, subjects received priming pulses and were shaped to approach and press the lever. Current intensity was initially set at 20 μA, and was raised by 5 μA every 3 min until vigorous self-stimulation occurred (operationally defined as ≥3 bar presses per min). Each subject's optimal current and pulse duration for eliciting maximal bar pressing rates was recorded and used on a subsequent test to determine bar pressing rates.

2.3. Testing experiment 2: place conditioning procedure

Place conditioning was conducted using an unbiased procedure as described elsewhere [28]. The place conditioning apparatus consisted of shuttle boxes made of Plexiglas (30 cm × 50 cm × 30 cm), each equipped with a loose-fitting wire mesh lid. One side of the shuttle box was white with a textured floor (i.e. small ridges and valleys approximately 0.5 mm tall and wide), while the other side was black with a smooth floor. During conditioning sessions, a slide partition divided the two sides, creating two different conditioning "environments". During the testing sessions of experiment 1, the partition was replaced with a 20 cm × 15 cm door that allowed the animal unrestricted access to either environment. The light level (∼10 lx) on each side was matched with a portable lamp suspended over the test chamber.

Before the start of conditioning, all rats were habituated to the place preference chamber for 15 min, and during this free exploration phase, the compartment preferences for each animal were determined. Two conditioning session was conducted, with the vehicle pairing consisting of 0.9% saline in the black side of the chamber and the drug injection consisting of 100 ng Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol (DAMGO; Sigma, USA) pairing in the white side. Each conditioning session lasted 30 min. USVs were recorded during both the drug and vehicle conditioning trials. Each conditioning session had at least 2–3 days separating the sessions that were counterbalanced for drug and vehicle injection order. USVs were recorded during the conditioning sessions. All injections were 500 nl in volume and were injected over 1 min into the brain and the injection cannulae was left in place for an additional 30 s before removal. After injections, rats were immediately confined to the white compartment following drug injections or the black compartment following vehicle injections. After place preference conditioning, rats were again allowed free access to both chambers for 15 min to test for post-drug conditioning preference. After histological analysis, only animals that had cannulae injection tips that were at least partially contained within the VTA were used for analysis ($n = 18$). Histological analysis of placements was done blind with respect to the vocalization eliciting or rewarding effects of the injection site.

2.4. Experiment 3: lesions of the mesolimbic dopamine system and pharmacological tests

2.4.1. Induction of USVs by heterospecific hand play (i.e. "tickling")

Tickling consisted of vigorous whole-body playful simulation that included repeated pinning of the animal resembling rough-and-tumble play. For all animals, the tickling was done with one hand and consisted of scaled-down rapid finger and hand movements commonly used in human tickling (see [25]). Tickling was conducted in a 45 cm × 35 cm × 20 cm opaque plastic box without

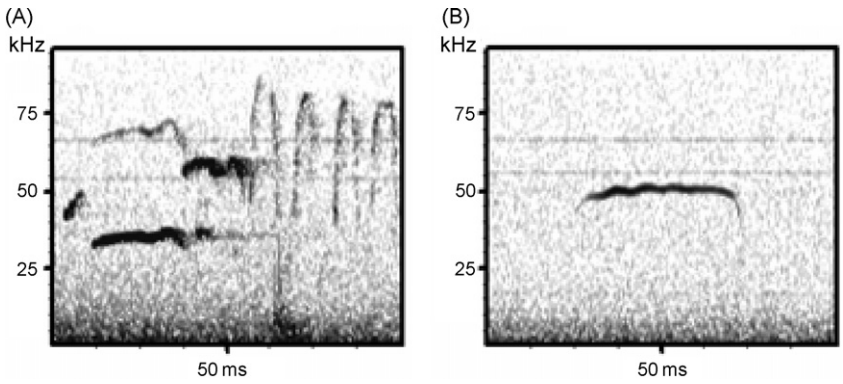

Fig. 1. (A) An example frequency modulated 50-kHz call, which contains a step component (~35-kHz) and/or more commonly a trill component (~70-kHz). (B) An example of a flat 50-kHz call which contains neither ~35-kHz step or ~70-kHz trill components.

bedding. Although the stimulation was rapid, brisk, and assertive, care was taken not to threaten the animal. The test chamber was divided into four equal size quadrants, and a line cross was counted when the animal crossed both forepaws over a line (which was used as an index of locomotor activity).

2.4.2. Lesions

After 3 days of habituation to tickling, animals that showed high levels of 50-kHz USVs in response to tickling (greater then 50 USVs/2 min) were used for these studies. 6-OHDA lesions ($n = 11$) were performed in an identical manner to Ref. [1], with the exception that isoflurane was used as anesthetic in the current study. In brief, animals were pretreated with desipramine and pargyline (15 and 50 mg/kg, i.p., respectively; Sigma, USA) 30 min before surgery. Eight μg 6-OHDA (Sigma, USA) dissolved in 4 μl of vehicle (0.9% NaCl solution with 0.1 mg/ml ascorbic acid) was infused in each posterior lateral hypothalamus (stereotaxic coordinates from bregma: −4.0 mm AP, ±1.8 mm ML, 8.3 mm DV, flat brain) using a Hamilton syringe fitted with a 30 gauge needle over 8 min. Control animals ($n = 11$) received vehicle injections. Animals were assigned to either the lesion group ($n = 8$) or the sham group ($n = 7$) matched for levels of 50-kHz USVs as exhibited on the third habituation day. Animals in the 6-OHDA lesion group were allowed to recover for 1-week post-surgery before the start of testing, and were tube fed two to three times a day with 5 ml per infusion (10–15 ml/day) of Ensure Plus (Abbott, USA) throughout recovery and testing.

Electrolytic lesions ($n = 8$) consisted of lowering 0-size insulated insect pin (with a 1 mm uninsulated tip exposure) bilaterally into the VTA through which 500 μA anodal DC current was passed for 30 s bilaterally into the VTA (stereotaxic coordinates from bregma: −6.0 mm AP, ±0.5 mm ML, −8.6 mm DV). Sham lesions ($n = 7$) were performed in an identical manner except that no current was passed through the electrode. Animals in the electrolytic lesion group were allowed to recover for 1-week post-surgery before the start of testing. If animals lost more than 5% of pre-surgery body weight, they were tube fed with a 10% glucose solution (5 ml, twice per day) until the animals no longer showed sustained weight loss.

Lesion and sham animals received 3 consecutive days of tickling 1 week after surgery for the 6-OHDA experiment, and 1 week and 1 month after surgery for the electrolytic experiment. Only the last test sessions of the 3 consecutive days of testing at the 1-week and 1-month time points were used for analysis.

2.4.3. Dopamine assay

Samples of nucleus accumbens were dissected from fresh tissues and maintained at −80 °C until assay. Prior to analysis, samples were thawed and sonicated in 1.5 ml screwtop microfuge tubes (Sarstedt, USA) containing 750 μl of *N*-HCL and 1.5 nmol of [$^2H_4$]dopamine. The samples were centrifuged at 4 °C for 40 min at 25,000 × *g*. The supernatants were dried overnight in a Savant concentrator. To the dried samples, 50 μl of ethyl acetate and 100 μl of pentafluoropropionic anhydride were added and the samples heated at 80 °C for 1 h samples were subsequently transferred to autosampler vials. Samples were analyzed using selected ion monitoring of the [M-147]$^-$ ions for dopamine (443) and for [$^2H_4$]dopamine (447) as described previously [34], except that the reagent

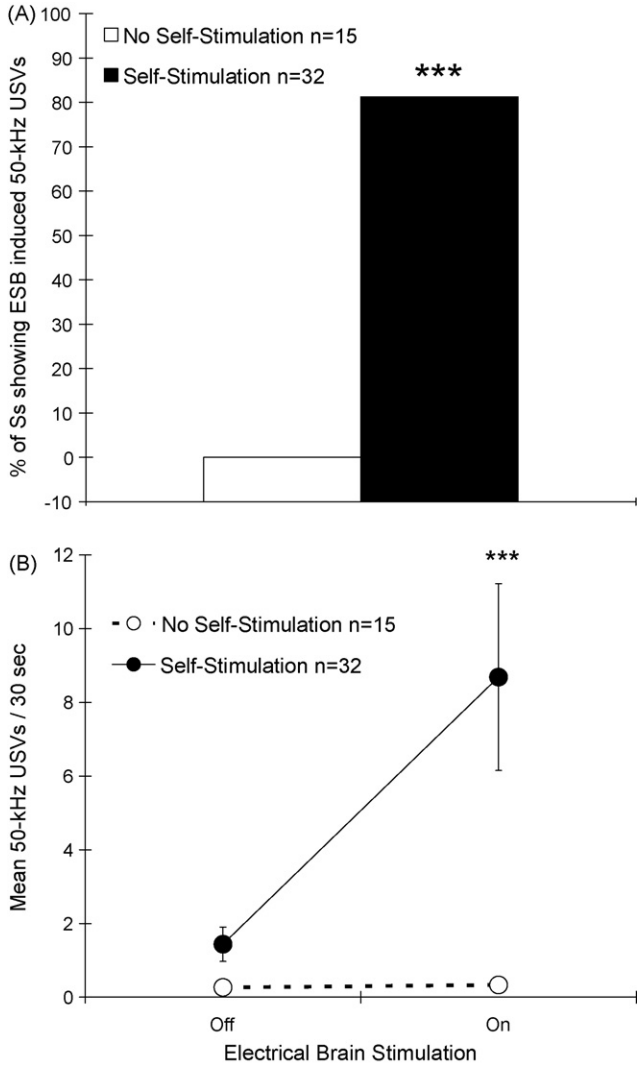

Fig. 2. (A) Percentage of animals (Ss) showing repeatable electrical brain stimulation-induced 50-kHz USVs that also show self-stimulation behavior. $^{***}P < .001$, two-tailed between subject *t*-test. (B) Mean ± S.E.M. rates of 50-kHz USVs in response to electrical brain stimulation (120 μA, 10 s, bipolar stimulation) that has been shown to maximize dopamine release [23] in animals that showed self-stimulation or did not show self-stimulation. $^{***}P < .005$, two-tailed within subject *t*-test.

gas was ammonia. An Agilent bench-top GC-MSD (HP6890/MSD5973) system was utilized: source (150 °C), quadrapole (150 °C), interface (320 °C), injector (250 °C), emission (150 μA) and eV (150). The injection port liner was packed with 2% SP-2250 on 100/200 Supelcoport (Sigma, USA), serving as a precolumn. Aliquots of 1 μl were injected splitless. The HP-5 capillary column (25 m, 0.25 mm i.d. and 0.25 μm thickness) was held at 130 °C for 1 min followed by 30 °C gradient with a carrier gas (He) flow of 1.2 ml/min. The retention time for dopamine was 4.2 min.

2.4.4. Pharmacological tests of tickled animals

After 3 days of habituation to tickling, animals that showed high levels of 50-kHz USVs in response to tickling (greater then 50 USVs/2 min) were

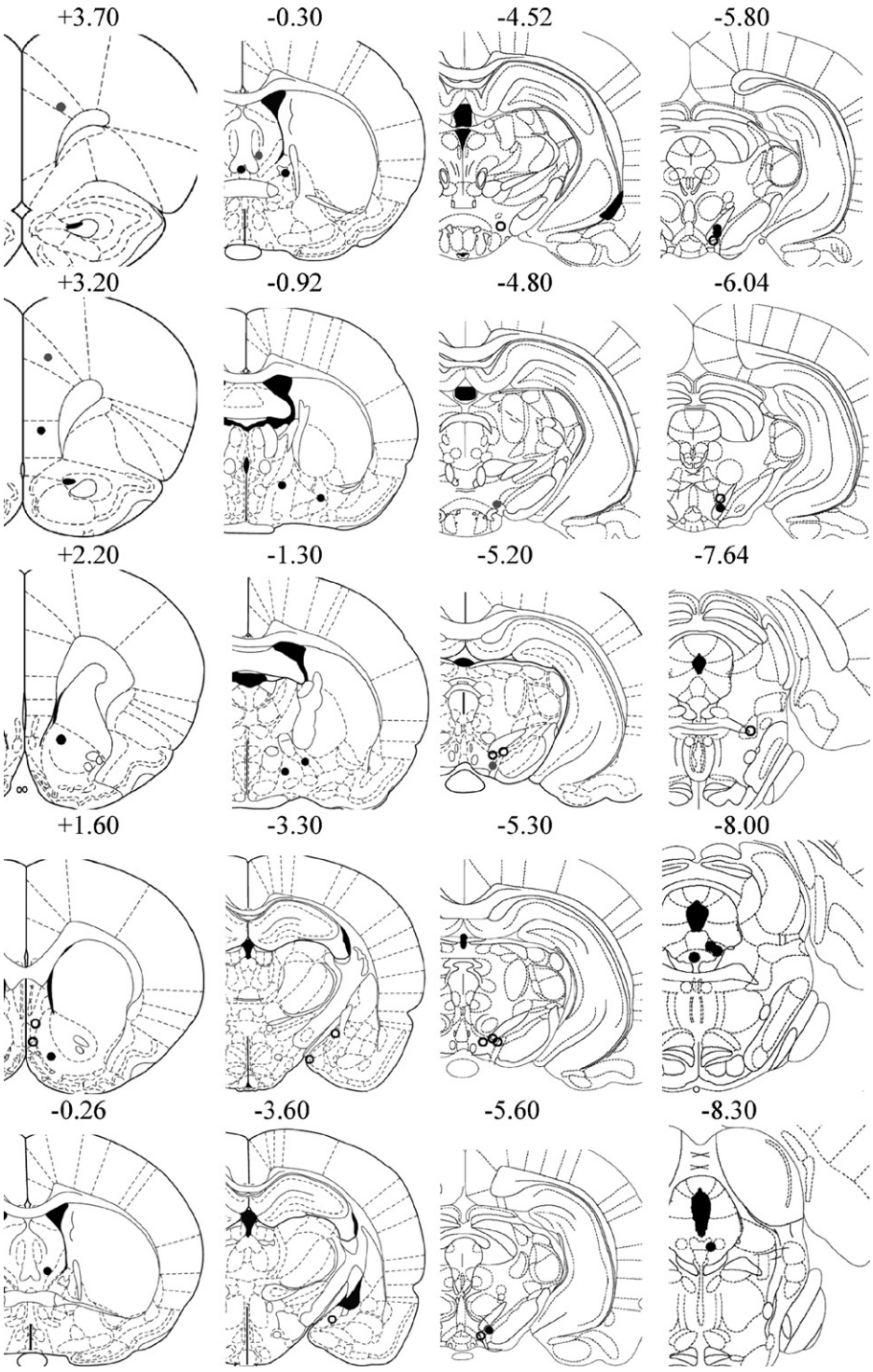

Fig. 3. Anatomical location of electrode tips in the rat brain from experiment 1. Anatomical plates adapted from Paxinos and Watson [26]. All coordinates are mm from bregma. Open circles represent placements that did not yield ESB-induced 50-kHz USVs. Gray circles represent placements that yielded repeatable ESB-induced 50-kHz USVs in response to more then one stimulation. Black circles represented placements that elicited ESB-induced 50-kHz consistently (at least 5 out of 6 consecutive stimulations and in most cases across test days) at a fixed stimulation threshold in which vocalizations were not consistently present below the threshold, and were present above the threshold.

used for these studies. Either flupenthixol, a D1/D2 antagonist ($n = 20$; 0 or 0.8 mg/kg, i.p.; Sigma, USA) in DMSO vehicle were administered 2.5 h as in Ref. [18] or diphenhydramine, a H1 antagonist ($n = 20$; 0 or 40 mg/kg i.p.; Sigma, USA) in 0.9% saline vehicle 15 min before the start of testing. Drug and vehicle administration was counterbalanced within subjects. Drug and vehicle test days were separated by a single test day where rats received 2 min of tickling without injection.

2.4.5. Pharmacological tests with electrical brain stimulation-induced vocalization

Animals were implanted with bipolar electrodes into the ventral tegmental area ($n = 4$), nucleus accumbens ($n = 3$), or bed nucleus of the stria terminalis ($n = 1$) and were shown to have reliable and repeatable electrical brain stimulation induced 50-kHz USVs. The optimal current threshold for eliciting 50-kHz USVs was determined for each animal using 60 Hz sine wave current. Brain stimulation sessions were conducted in a 34 cm × 23 cm × 32.5 cm translucent box using a commutator (Plastics One, USA) and consisted of a single 2 min session each day in which animals received 10 s of continuous brain stimulation 40 s and 80 s into the session. 0.8 mg/kg flupenthixol or vehicle was administered i.p. 2.5 h before the start of the test session as in Ref. [18]. Drug and vehicle injections were counterbalanced within animals, and all subjects received a single re-habituation session between the two test days.

2.4.6. Ultrasonic vocalization recording and analysis

USVs were recorded from the high frequency output of a model D980 bat detector (Pettersson Elektronik AB, Sweden) and recorded digitally with a Fostex Fr-2 field recorder (USA). Sonographic analysis of USVs was done in a blind manner with SAS lab Pro (Avisoft Bioacoustics, Germany) by a trained observer. Ultrasonic USVs were divided into three separate categories for analysis (1) frequency modulated (FM) 50-kHz USVs containing trill and/or step components, (2) flat 50-kHz USVs (Fig. 1), and (3) 22-kHz USVs.

2.4.7. Histological verification of electrode and cannulae placement

At the conclusion of behavioral testing, animals that had received surgery were sacrificed with carbon dioxide and their brain was rapidly removed. Brains were placed into a 30% sucrose–10% formaldehyde–0.9% saline solution (w/v), and stored at 4 °C for at least 1 month before slicing. Brains were then frozen and sliced into 50 μM coronal sections with a freezing microtome. Sections containing the tips of the electrodes were mounted on microscope slides, and both electrode and cannulae tips were localized by projecting the magnified slides onto figures derived from the atlas of Paxinos and Watson [26]. Histological reconstruction was done in a blind manner with respect to the behavioral effects of ESB or chemical microinjections.

3. Results

3.1. Experiment 1

All animals tested that showed repeatable ESB-induced 50-kHz USVs also showed self-stimulation ($n = 26$). The minimum self-stimulation rate was set at 3 bar presses/min, which is significantly greater then the free operant bar-pressing rate (mean ± S.E.M.) of 0.4 ± 0.2 bar presses/min ($t(9) = 13.96$, $P < .0001$). All animals that showed bar pressing rates greater the 3 per min were categorized as self-stimulating animals, and subsequent analysis treated self-stimulation as a dichotomous (self-stimulators versus non-self-stimulators) variable as opposed to a continuous variable (self-stimulation rate). Eighty-one percent of all self-stimulating animals showed repeatable ESB-induced 50-kHz USVs, whereas none of the animals that failed to show self-stimulation behavior exhibited repeatable ESB-induced 50-kHz USVs ($\chi^2 = 27.28$, $P < .0001$; Fig. 2A). Rates of 50-kHz USVs were also different between these groups ($t(14) = 0.33$, $P > .05$; Fig. 2B). The distribution 50-kHz USVs in response to electrical brain stimulation was not parametrically distributed (i.e. many 0 values for the non-self-stimulation group, see Fig. 2B), therefore the data underwent a square root transformation before analysis. Self-stimulating animals also showed a significant increase in 50-kHz USV rates to experimenter delivered ESB compared to baseline, whereas non-self-stimulating animals did not show this effect, as indicated by a significant interaction between self-stimulation status (self-stimulator or non-self-stimulator) and stimulation (on or off) conditions ($F(1, 90) = 5.63$, $P < .05$). The increase in 50-kHz USV rates in response to experimenter delivered stimulation was also greater for self-stimulating rats compared to non-self-stimulating rats ($t(45) = 3.72$, $P < .001$; Fig. 2B). Histological reconstructions of electrode placements are summarized in Fig. 3. USVs during self-stimulation tests could not be quantified sonographically due to electronic noise generated by the mechanical barpress counter, the turning on and off of the brain stimulator, and the mechanical noise generated by the lever presses (which for many animals were sustained at a rate of ∼1 per s).

3.2. Experiment 2

Animals were classified as DAMGO Vocalizers if they exhibited twice as many 50-kHz USVs in response to DAMGO administration in the first 5 min as compared to vehicle injections, or exhibited more 50-kHz USVs during both the first and second 5 min blocks for the DAMGO compared to vehicle injection. The peak drug effect occurred within the first 5 min after DAMGO injection, with 71% of all DAMGO-elicited vocalizations occurring during the first 10 min of each 25 min test session. Animals that did not make this criterion were referred to as DAMGO Non-Vocalizers. DAMGO Vocalizers animals showed a greater increase in 50-kHz USVs in response to

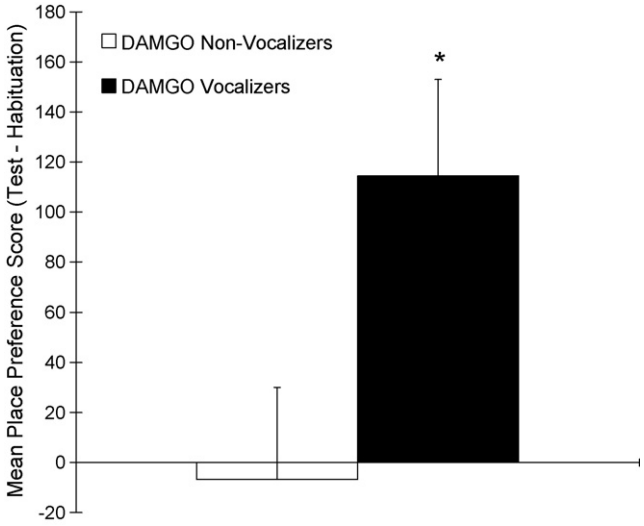

Fig. 4. Mean ± S.E.M. increase in time (s) spent on drug paired side after drug conditioning compared to pre-conditioning in animals that showed more USVs in response to drug injection (DAMGO Vocalizers) compared to DAMGO Non-Vocalizers. $^{*}P < .05$ within subject t-test, two-tailed comparing pre- vs. post-conditioning.

DAMGO compared to vehicle injection during the first 10 min of testing compared to DAMGO Non-Vocalizers (Mann–Whitney U, $P < .05$). Using these grouping, DAMGO Vocalizers showed significant place preference for the environment paired with drug injection ($t(5) = 3.0$, $P < .05$), whereas DAMGO Non-Vocalizers showed no significant place preference for the environment paired with drug injection ($t(10) = 0.2$, $P > .05$; Fig. 4). Histological reconstruction of cannulae placements are summarized in Fig. 5.

3.3. Experiment 3

3.3.1. Electrolytic VTA lesions

As depicted in Fig. 6, animals receiving electrolytic lesion of the VTA showed a significant reduction in frequency modulated 50-kHz USVs compared to sham operated animals 1 month after surgery ($t(13) = 2.5$, $P < .05$), while flat 50-kHz USVs and 22-kHz USVs did not significantly differ between lesion and sham groups (all P's $> .05$). Line crosses and weight loss were not significantly different comparing lesion versus sham animals (all P's $> .05$; Fig. 7). Histological analysis of VTA lesions are reported in Fig. 8. Dopamine levels (mean ± S.E.M. as percent of sham lesion levels) in VTA lesioned animals were significantly reduced in the basal forebrain sample that included the nucleus accumbens (65.9% ± 11.0; $t(13) = 2.7$, $P < .05$), but not in the dorsal striatum (i.e. caudate nucleus (95.3% ± 10.1; $P > .05$) or the olfactory bulbs (77.5% ± 6.6; $P > .05$).

3.3.2. 6-Hydroxydopamine lesions

As depicted in Fig. 9, 6-OHDA lesioned animals showed a significant reduction in frequency-modulated (FM) 50-kHz USVs compared to sham operated animals 1 week after surgery ($t(32) = 2.8$, $P < .01$), while flat 50-kHz USVs and 22-kHz USVs did not significantly differ between lesion and sham groups

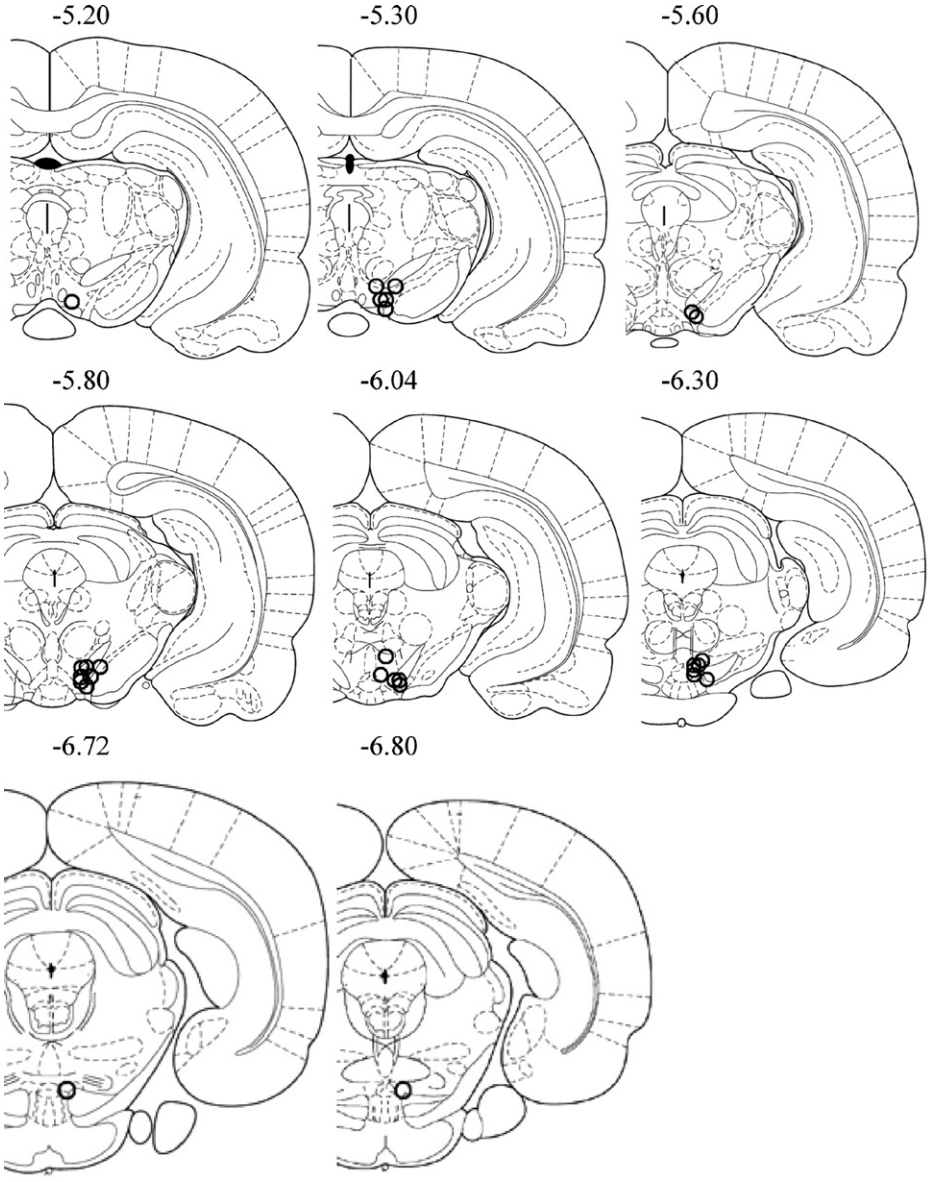

Fig. 5. Anatomical location of cannulae tips (circles) in the rat brain in experiment 2. All coordinates are mm from bregma. Anatomical plates adapted from Paxinos and Watson [26].

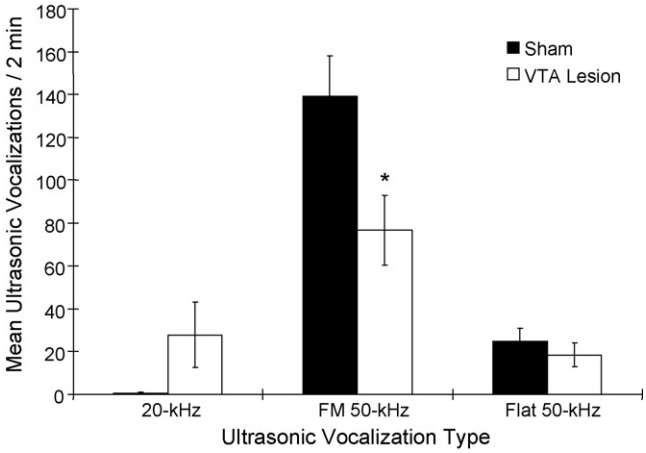

Fig. 6. Mean (±S.E.M.) USVs in response to 2 min of heterospecific play (i.e. "tickling") 1 month after receiving either electrolytic lesions of the ventral tegmental area or sham lesions. $^{*}P < .05$, between subjects *t*-test, two-tailed.

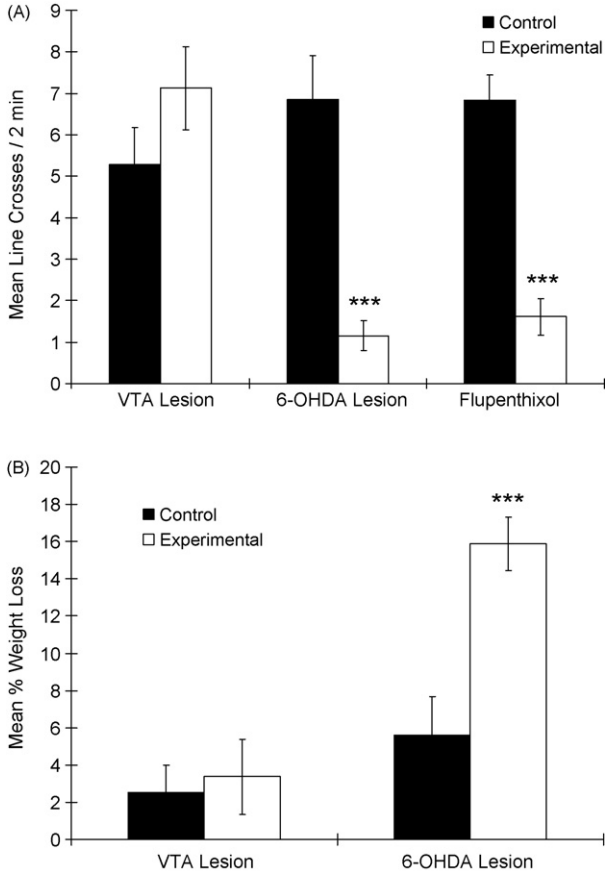

Fig. 7. Mean (±S.E.M.). (A) Line crosses in response to heterospecific play (i.e. "tickling") in animals receiving electrolytic lesions of the ventral tegmental area, 6-OHDA lesions of the medial forebrain bundle, or systemic injections of flupenthixol (with each group being compared to their control conditions). (B) Percent weight loss at the end of testing as compared to pre-surgery weight in ventral tegmental area (VTA) and 6-OHDA lesions of the medial forebrain bundle as compared to their sham lesioned controls. $^{***}P < .001$, between subjects *t*-test, two-tailed.

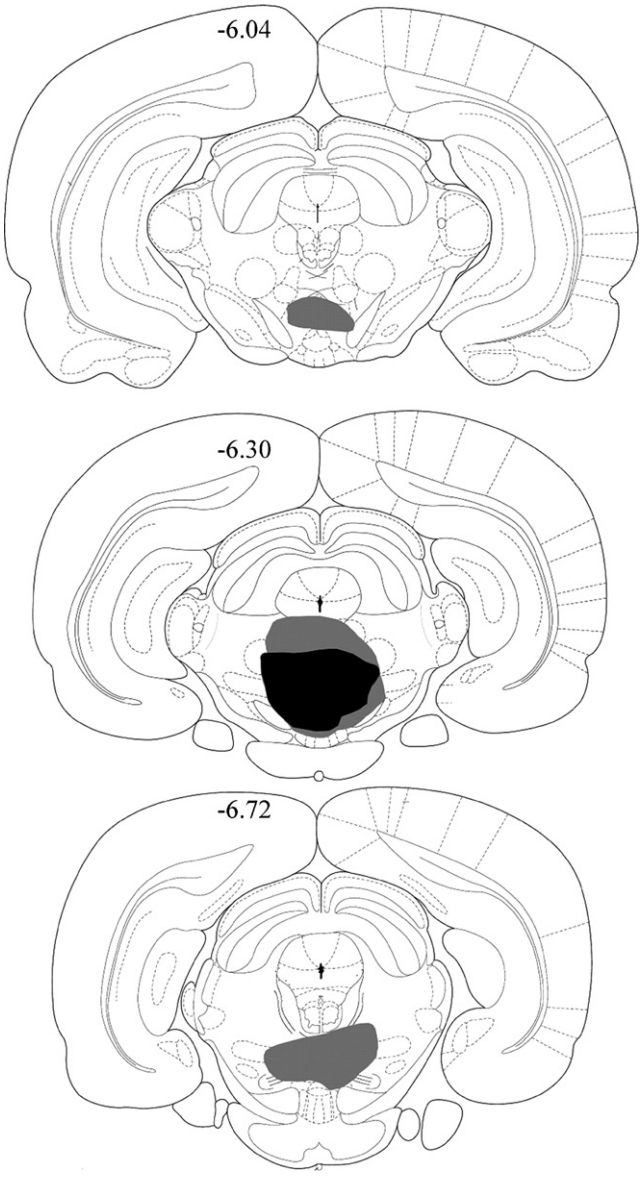

Fig. 8. Anatomical localization of bilateral ventral tegmental electrolytic lesions based on the atlas of Paxinos and Watson [26]. Black shading represents lesion area shared by all of the animals, and gray shadings represents area shared by only a subset of the animals. All coordinates are mm from bregma.

(all *P*'s > .05). Line crosses were reduced in 6-OHDA lesioned animals ($t(32) = 5.8$, $P < .001$) and weight loss was greater in 6-OHDA lesioned animals ($t(32) = 4.2$, $P < .001$) as compared to sham lesioned control animals 1 week after surgery (Fig. 7). Dopamine levels (mean ± S.E.M. as percent of sham lesion levels) in 6-OHDA lesioned animals were significantly reduced in the basal forebrain sample that included the nucleus accumbens (8.5% ± 2.4) and in the dorsal striatum (10.8% ± 1.6; all *P*'s < .0001).

3.3.3. Pharmacological analysis

As depicted in Fig. 10A, flupenthixol reduced FM 50-kHz USVs compared to vehicle treatment during tickling ($t(17) = 2.34$, $P < .05$), but not flat 50-kHz or 22-kHz USVs (all *P*'s > .05). Locomotor activity as measured by line crosses was

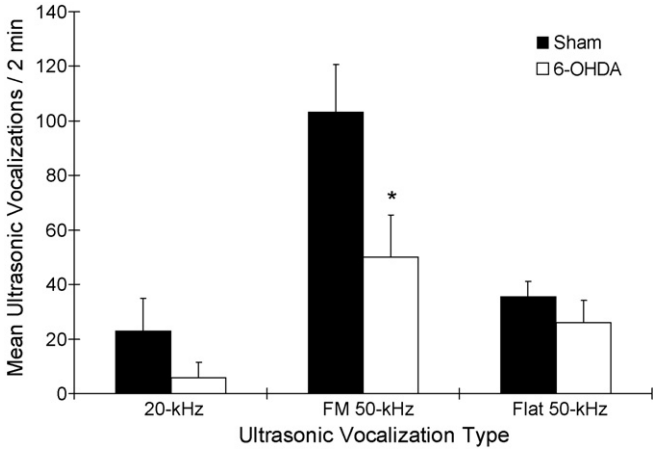

Fig. 9. Mean number of (±S.E.M.) frequency modulated 50-kHz, flat 50-kHz and 22-kHz USVs in response to 2 min of heterospecific play (i.e. "tickling") 1 month after receiving either 6-hydroxydopamine (6-OHDA) lesions of the medial forebrain bundle or sham lesions (Sham). $^{*}P < .01$, between subjects *t*-test, two-tailed.

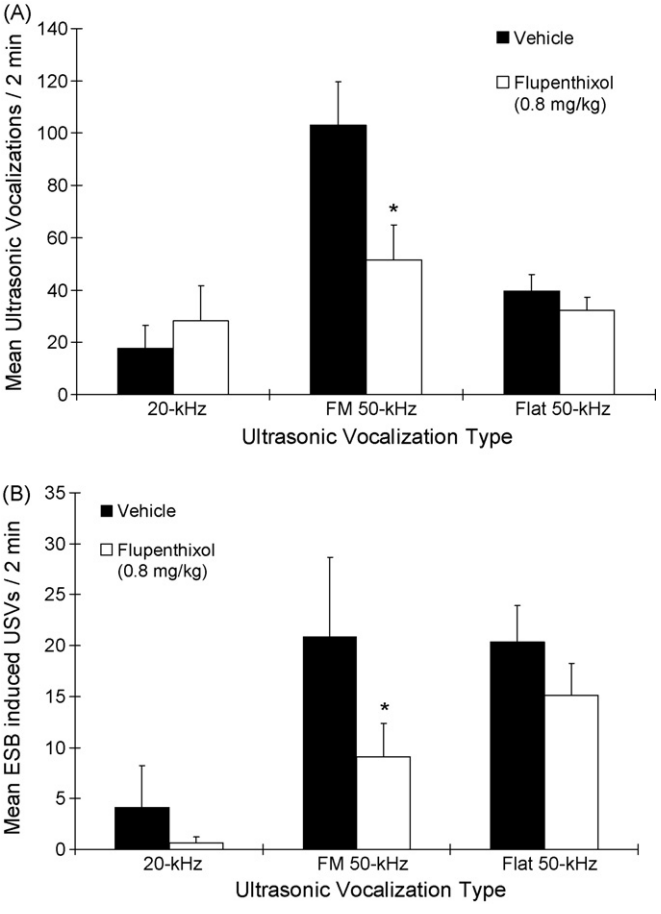

Fig. 10. (A) Mean (±S.E.M.) number of frequency modulated 50-kHz, flat 50-kHz and 22-kHz USVs in response to 2 min of heterospecific play (i.e. "tickling") in response to pretreatment with the D1/D2 dopamine antagonist flupenthixol (0.8 mg/kg, i.p.) or vehicle. (B) Mean (±S.E.M.) number of USVs in response to 2 min of electrical brain stimulation for animals with electrode placements in either the accumbens, bed nucleus of the stria terminalis or ventral tegmental area in response to pretreatment with the D1/D2 dopamine antagonist flupenthixol (0.8 mg/kg, i.p.) or vehicle. $^{*}P < .05$, within subject *t*-test, two-tailed.

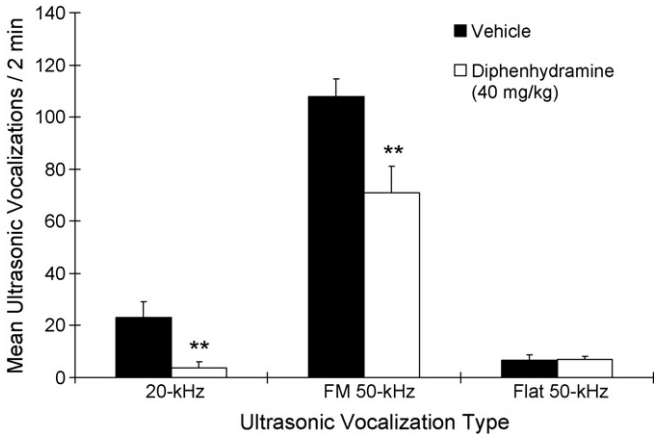

Fig. 11. Mean (±S.E.M.) number of USVs in response to 2 min of heterospecific play in response to pretreatment with the H1 histamine antagonist (i.e. "tickling") diphenhydramine (40 mg/kg, i.p.) or vehicle. $^{**}P < .01$, within subject *t*-test, two-tailed.

also reduced by flupenthixol compared to vehicle treated animals ($t(17) = 6.47$, $P < .0001$; Fig. 7). Flupenthixol also reduced FM 50-kHz USVs compared to vehicle treatment during brain stimulation ($t(7) = 2.4$, $P < .05$), but not flat 50-kHz or 22-kHz USVs (all P's $> .05$; Fig. 10B) Compared to vehicle treatment, diphenhydramine reduced FM 50-kHz USVs ($t(19) = 3.19$, $P < .005$), as well as 22-kHz USVs ($t(19) = 3.11$, $P < .01$), but not flat 50-kHz USVs ($P > .05$; Fig. 11). Locomotor activity as measured by line crosses was not significantly altered by diphenhydramine (mean ± S.E.M.; 5.6 ± 1.5) compared to vehicle (8.0 ± 1.1) treated animals ($P > .05$).

4. Discussion

These experiments demonstrated, that electrical or chemical stimulation of the brain that elicits 50-kHz USVs is rewarding to rats. This provided further support that 50-kHz USVs are positively related to reward, and that the neural circuit for the generation of 50-kHz USVs closely overlaps that of electrical brain self-stimulation and drug reward and hence may be reflective of induction of positive affective states within the brain [6,17,30]. The results of experiment 1 provided a functional neuroanatomical substrate for 50-kHz USVs and confirmed that 50-kHz USVs are related to reward, given that all electrode sites that elicited repeatable 50-kHz USVs supported self-stimulation. These results are consistent with the results of Jürgens [13], who showed that the brain stimulation-elicited trill vocalizations in squirrel monkeys are positively correlated to self-stimulation in many of the same brain regions as tested in this study. A common variant of 50-kHz USVs involves a FM trill component, and both the rodent 50-kHz USV with trill components and the squirrel monkey trill call are both related to positive appetitive behavior [14,32].

The results of experiment 2 demonstrated that drugs with rewarding properties that are focally injected into the VTA are primarily rewarding in animals that exhibited elevated levels of 50-kHz USVs in association with these injections. Given that the rewarding effect of opiates in the VTA, as well as electrical brain

stimulation of this region, are thought to partially depend on the mesolimbic dopamine system, we evaluated whether ESB-induced 50-kHz USVs could be attenuated by the dopamine receptor antagonist, flupenthixol. That proved to be the case, but flupenthixol only decreased rates of FM 50-kHz USVs (which includes trill USVs). These findings are consistent with previous demonstrations that injections of amphetamine into the accumbens shell produce a dramatic ∼35-fold elevation in rates of 50-kHz USVs [3,31].

These results also provide the first demonstration that disruption of the mesolimbic dopamine system either by lesions or pharmacological blockade specifically reduced levels of FM 50-kHz USVs that are commonly emitted in response to rewarding and positive affective stimuli. The same manipulations do not effect flat 50-kHz USVs nor aversive 22-kHz USVs [6]. We also found that the locomotor and appetite deficits associated with dopamine depletion were not the main causes of the decreases in frequency modulated 50-kHz USVs seen in this study. This is because electrolytic lesions of the ventral tegmental area reduced frequency modulated 50-kHz USVs in a similar manner as 6-OHDA lesions and D1/D2 receptor antagonist flupenthixol, but did not disrupt locomotor activity or body weight at the time of testing.

Histamine has been shown to increase rates of positive affective vocalizations when injected directly into the squirrel monkey brain [20]. Here, pharmacological antagonism of the histamine H1 receptor by systemic diphenhydramine reduced both reward related FM 50-kHz USVs and aversive 22-kHz USVs, with the latter effect being consistent with the anxiolytic actions of diphenhydramine [10]. This finding also demonstrates that, despite the relatively low levels of 22-kHz USVs and flat 50-kHz USVs observed in our tickling procedure, 22-kHz USVs could still be significantly reduced from those already low levels.

Although all animals used in these studies were adult female Long Evans rats, it is unlikely that variability in ultrasonic vocalization rates and operant behavior due to estrous cycle changes could explain the findings of this study. While levels of USVs do vary during the estrous cycle, this effect is dependent on to the presence of a hormonally intact male rat [21]. In the experiments reported here, all females were virgins and were not exposed to male odors during testing given that the equipment used in these studies were used only by female rats. While operant responding for ESB in some brain areas has been shown to vary modestly across estrus cycle [29], the presence or absence of self-stimulation behavior in the current study remained quite stable across test days. Also, in the present studies the experimenter was blind to estrous condition of the animal. Therefore, it is probable that any variability induced by the estrous state of the animal would have been evenly distributed across conditions.

Future studies will examine the relationship between the mesolimbic dopamine system and 50-kHz USVs by examining the relationship between frequency modulated 50-kHz USVs and the in vivo release of dopamine, and attempt to record ultrasonic vocalizations during self administration of both electrical brain stimulation and drugs of abuse. These studies will further elucidate the neurobiology of positive affect and addictive rewards.

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