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High-Frequency Ultrasonic Vocalizations Index Conditioned Pharmacological Reward in Rats

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KNUTSON, B., J. BURGDORF AND J. PANKSEPP. *High-frequency ultrasonic vocalizations index conditioned pharmacological reward in rats.* PHYSIOL BEHAV **66**(4) 639–643, 1999.—We have proposed that short (<0.5 s), high-frequency (~50 kHz) ultrasonic vocalizations ("50-kHz USVs") index a positive affective state in adult rats, because they occur prior to rewarding social interactions (i.e., rough-and-tumble play, sex). To evaluate this hypothesis in the case of nonsocial stimuli, we examined whether rats would make increased 50-kHz USVs in places associated with the administration of rewarding pharmacological compounds [i.e., amphetamine (AMPH) and morphine (MORPH)]. In Experiment 1, rats made a greater percentage of 50-kHz USVs on the AMPH-paired side of a two-compartment chamber than on the vehicle-paired side, even after statistical correction for place preference. In Experiment 2, rats made a higher percentage of 50-kHz USVs on the MORPH-paired side than on the vehicle-paired side, despite nonsignificant place preference. These findings support the hypothesis that 50-kHz USVs mark a positive affective state in rats and introduce a novel and rapid marker of pharmacological reward. © 1999 Elsevier Science Inc.

Reward Vocalization Rat Amphetamine Morphine

CAREFUL ethological studies have revealed that rats make USVs in many different social and emotional situations (15). These USVs vary in frequency and duration, but two typical variants can be distinguished in adults: long low vocalizations (>0.5 s, ~22 kHz; hereafter, "22-kHz USVs") and short high vocalizations [<0.5 s, ~50 kHz; hereafter, "50-kHz USVs;" (13)]. These two types of vocalizations may be produced by separable physiological mechanisms (4), and may evoke different social responses from other rats [e.g., (27)].

Several investigators have verified that aversive experiences predominantly evoke 22-kHz USVs. For instance, rats make 22-kHz USVs in the presence of predators (3), during submissive behaviors in a fight (8), and in anticipation of punishment (22). Thus, some investigators have postulated that 22-kHz USVs mark a negative affective state (9). This hypothesis is consistent with the observation that administration of anxiolytics reduces 22-kHz USVs in aversive situations (24,25).

Contexts that elicit 50-kHz USVs have been less extensively characterized. Rats make 50-kHz USVs during appetitive aspects of sexual behavior (2) and other rewarding social encounters such as rough-and-tumble play (12). These observations have led us to hypothesize that 50-kHz USVs mark a valenced state in rats, which involves both high arousal and the expectation of a hedonically positive outcome. Drawing from early place preference research (14) as well as an extensive body of human psychometric research (26), we will call this state "positive affect." In support of this hypothesis, we have found that direct administration of the dopaminergic agonist amphetamine (AMPH), which is thought to enhance positive affect in low doses, increases 50-kHz USVs at a dose of 1.0 mg/kg (but not 0.5 or 2.0 mg/kg) while simultaneously reducing 22-kHz USVs (11). Further, we have recently observed that rats make 50-kHz USVs in anticipation of brain stimulation of mesolimbic dopaminergic pathways associated with reward (6).

If short 50-kHz USVs mark a positive affective state in rats, then rats should make more of these vocalizations in places where they have previously had rewarding pharmacological experiences. Place-preference studies have fairly consistently indicated that rats prefer to inhabit environments associated both with prior amphetamine (AMPH) and morphine

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(MORPH) exposure [e.g., (16,20)]. In the following experiments, we examined rats' production of 50-kHz USVs in environments previously paired with AMPH and MORPH administration.

EXPERIMENT 1

Experiment 1 was designed to determine whether an environment associated with AMPH treatment would facilitate 50-kHz USVs. We were also interested in determining whether USVs versus place-preference measures would exhibit comparable sensitivity as markers of prior AMPH treatment.

Method

Subjects. Subjects were 30 110-day-old hooded Long–Evans rats (20 males and 10 females) bred at the Bowling Green State University animal facility. Animals were individually housed after weaning at 21 days of age and received free access to food and water thereafter. Lighting was maintained on a 12:12 light:dark cycle, and testing occurred during the second half of the light phase.

Apparatus. Animals were tested in a 48 × 19 × 28-cm shuttlebox, which was divided in half by an aluminum partition with a 15 × 20-cm doorway. A soundproof enclosure shielded the shuttlebox from peripheral noise and distractions during testing. Both visual and tactile cues distinguished the left and right "chambers" of the shuttlebox. The left chamber had horizontal stripes on the walls and a wire mesh floor, whereas the right chamber had vertical stripes on the walls and a floor composed of parallel bars spaced 1 cm apart. During testing, a Commodore computer recorded the number of shuttles from one chamber to the other via photocells that were placed 5 cm away from the divider in each chamber, yielding a measure of time spent in each. A coder recorded 50-kHz USVs on line from outside the soundproof enclosure via remote headphones connected to two Mini-3 Bat Detectors (Ultra Sound Advice, London), which were installed in the ceiling of each chamber 5 cm away from and facing the back walls. Both detectors were tuned to 50 kHz (sensitivity range = 40–60 kHz).

During conditioning, a metal door separated the adjacent chambers, so that the animal was confined to one chamber or the other. During testing, the door was removed to allow free access to both chambers. Throughout the 5-min test session, coders registered USVs as occurring either in the right or left chamber. By listening to the right bat detector in the right earphone and the left bat detector in the left earphone and watching the rat through the window of the soundproof box, coders could localize a given subject's vocalization based on the location of the tip of its nose. For example, if a rat vocalized while its nose was in the right chamber (regardless of the position of the rest of its body), then the coder recorded a vocalization on the right side of the box.

Procedure. Prior to conditioning, animals were placed in the shuttlebox with the door open for a habituation period lasting 15 min, during which their baseline chamber preference (relative occupancy of the right versus the left chamber) was recorded. Subsequent conditioning lasted for 4 days, followed by testing on the fifth day. Twenty animals (14 males and 6 females) were randomly assigned to the experimental group and the remaining 10 (6 males and 4 females) comprised the control group. On the first day of conditioning, half of the experimental and control animals were placed in their nonpreferred chamber for 15 min, while the other half were placed in their preferred chamber (computed from baseline scores). On the second day, subjects were placed in the opposite chamber. This process was repeated on the third and fourth days, so that all animals received two exposures to both their preferred and nonpreferred chambers. Thus, animals received place conditioning according to a "biased" design (7), which necessitated the inclusion of a vehicle control group (23).

Experimental rats received 0.5 mg/kg i.p. injections of AMPH prior to exposure to the nonpreferred chamber and VEH injections prior to exposure to the preferred chamber. Dosage was selected based on a meta-analysis, which reported that the lowest AMPH doses to produce significant place preference fell between 0.3 and 1.00 mg/kg (1). Control rats, on the other hand, received i.p. VEH injections prior to exposure to both nonpreferred and preferred chambers.

Testing occurred on the fifth day, following conditioning. The door was removed from the shuttlebox, all animals received an i.p. injection of vehicle, were placed in their initially preferred side, and observed for 5 min. Unlike most place preference studies, we measured vocalizations and chamber occupancy only during the first 5 min of testing because we have found that rats vocalize most during the first 5 min of reintroduction to a familiar environment, after which vocalizations fall off rapidly (12).

Results and Discussion

We predicted that experimental rats would associate a positive affective state with the chamber in which they had received AMPH, and thus would emit more 50-kHz USVs in that chamber, in addition to spending more time there. 2 (AMPH versus VEH treatment) × 2 (male versus female) ANOVAs were conducted (a) on place preference and (b) on 50-kHz USVs with place preference as a covariate. Place preference was operationally defined as the percentage of the 5-min test period that a rat spent in the initially nonpreferred chamber (hereafter, the AMPH-paired chamber). 50-kHz USVs were similarly defined as percentage of vocalizations a rat made in the AMPH-paired chamber. Percentage scores were utilized rather than raw vocalization counts to minimize variance due to large individual differences we observed in the animals' baseline tendency to vocalize (though absolute counts are also reported in Table 1). Additionally, the covariance analysis on 50-kHz USVs allowed us to test both whether vocalizations were correlated with place preference, and whether shifts in chamber occupancy due to place preference would account for changes in vocalization.

The ANOVA for place preference revealed a significant main effect, indicating that experimental rats spent slightly more time in the AMPH-paired chamber (mean ± SEM: 53 ± 2%) than control rats, who received vehicle in both chambers [43 ± 4%; $F(1, 26) = 4.84$, $p < 0.05$; see Fig. 1a]. The AN-

TABLE 1

RAW COUNTS OF 50 kHz ULTRASONIC VOCALIZATIONS IN INITIALLY NONPREFERRED VERSUS PREFERRED CHAMBERS (MEAN ± SEM)

		Chamber	
		Nonpreferred	Preferred
Experiment 1	AMPH	114.25 ± 12.2	81.85 ± 7.6
	CONT	85.60 ± 16.0	96.70 ± 16.2
Experiment 2	MORPH	64.00 ± 17.2	33.25 ± 10.3
	CONT	41.12 ± 8.9	53.37 ± 10.3

COVA for 50-kHz USVs revealed a significant main effect of the covariate, indicating that place preference was correlated with 50-kHz USVs, $F(1, 25) = 14.88$, $p < 0.001$. But this analysis also yielded a significant main effect of treatment, $F(1, 25) = 5.34$, $p < 0.05$, independent of the covariate, indicating that experimental rats vocalized more in the AMPH-paired chamber (57 ± 2%) than did control rats (47 ± 3%), and that increased place preference could not account for differences in vocalizations (see Fig. 1b and Table 1). There were no significant effects of sex in either of the analyses. In sum, even though place preference was correlated with 50-kHz USVs, 50-kHz USVs provided a sensitive marker of prior AMPH treatment above and beyond place-preference effects.

EXPERIMENT 2

As with Experiment 1, Experiment 2 was designed to determine whether an environment associated with prior MORPH treatment would elicit increased 50-kHz USVs, even after correcting for place preference.

Method

Subjects. Subjects were 32 100-day-old hooded Long–Evans rats (16 males and 16 females), which were bred and cared for as described in Experiment 1.

Apparatus. Testing occurred in the same shuttlebox used in Experiment 1, with one modification. To gain more experimental control by narrowing the choice of discriminative stimuli, we removed the wire mesh from the left chamber, so that both floors were composed of bars spaced 1 cm apart. Thus, only visual stimuli distinguished the two chambers—one had vertically striped walls, while the other had horizontally striped walls. As before, a computer recorded chamber occupancy while a remote coder recorded 50-kHz USVs in each chamber on line via Bat Detectors installed in the ceiling.

Procedure. As in Experiment 1, rats were first placed in the shuttlebox with the door open for a 15-min habituation session, and their baseline chamber preference was recorded, after which the door between chambers was closed. Conditioning was similar to Experiment 1, but involved more sessions (five as opposed to two), to compensate for a possible drop in power due to the reduction of discriminative stimuli (5). Thus, rats were conditioned on Days 1–5 and tested on Day 6.

During each day of conditioning, the experimental group (eight males, eight females) received 1.0 mg/kg MORPH in the nonpreferred chamber but vehicle in the preferred chamber in counterbalanced order. As in Experiment 1, dose level was based on a meta-analytic report that the lowest doses of MORPH to elicit significant place preference ranged from 1.00–2.99 mg/kg (1). Control rats (eight males, eight females) received vehicle in both preferred and nonpreferred chambers on each of Days 1–5. On Day 6, the door was opened, all animals received an i.p. injection of vehicle, were placed in their initially preferred chamber, and chamber occupancy and 50-kHz USVs were recorded for 5 min.

Results and Discussion

As before, we predicted that experimental rats would associate a positive affective state with the chamber in which they received MORPH and, thus, would spend more time there and make more 50-kHz USVs there, even after taking place preference into consideration. 2 (MORPH versus VEH treatment) × 2 (male versus female) ANOVAs were conducted on (a) place preference and (b) 50-kHz USVs with place preference as a covariate. The ANOVA for place preference did not yield a significant main effect of treatment, indicating an absence of robust preference for the MORPH-paired side on the part of the experimental animals (see Fig. 2a). However, the ANCOVA for 50-kHz USVs revealed both a significant main effect of the covariate, indicating again that place preference was correlated with 50-kHz USVs, $F(1, 27) = 13.24$, $p < 0.001$, and a main effect of treatment, $F(1, 27) = 46.66$, $p < 0.001$, independent of the covariate, indicating that experimental rats vocalized substantially more in the MORPH-paired chamber

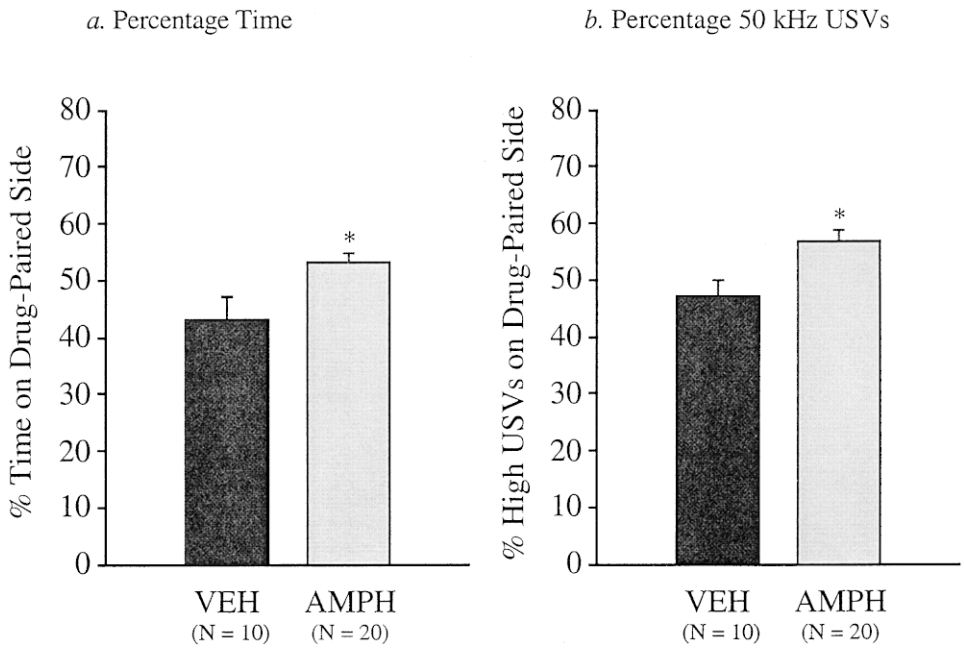

FIG. 1. Percentage time and 50-kHz USVs in AMPH-paired chamber (error bars = SEM, $*p < 0.05$).

(75 ± 4%) than did control rats (42 ± 3%). As in Experiment 1, changes in chamber occupancy due to place preference could not account for the observed difference in vocalizations (see Fig. 2b) and there were no significant effects of sex in either of the analyses. Thus, even in the absence of statistically significant place preference, the pattern of results found in Experiment 1 for 50-kHz USVs was replicated.

GENERAL DISCUSSION

These studies are the first to demonstrate that 50-kHz USVs may serve as a marker of pharmacological reward. Rats made more 50-kHz USVs in the chamber where they had previously received a rewarding compound (i.e., AMPH and MORPH), even after statistically controlling for differences in chamber occupancy. Within groups, the proportion of vocalizations that rats made on the drug-paired side was correlated with place preference, suggesting that the two measures indexed a similar construct. However, 50-kHz USVs typically predicted prior drug treatment more robustly than place preference, particularly in the case of MORPH. Despite adequate sample sizes, we may have found weak place-preference effects because we only measured the first 5 min of behavior when animals vocalize most, while other investigators typically measure 15–30 min of place preference behavior [see (5)]. Thus, it is not clear whether proportion of vocalizations is a more sensitive measure of prior drug treatment than place preference, or simply an earlier behavioral marker. Nonetheless, these findings are consistent with the hypothesis that 50-kHz USVs mark a positive affective state in adult rats, and further suggest that 50-kHz USVs may serve as a useful adjunct to more traditional place preference markers of pharmacological reward.

Despite their sensitivity and sponteneity, 50-kHz vocalizations currently have several limitations as standardized behavioral measures. First, as mentioned earlier, they follow a transient time course, falling off 5 min after reintroduction to a familiar environment. Second, their baseline expression tends to shift over days. We have observed that rats emit very few or none of these vocalizations when in a novel chamber, but high rates upon reintroduction across several days, after which vocalizations taper off after several more days. These shifting baselines may help to explain why the absolute amount of vocalization decreased in Experiment 2 (which included five conditioning trials), relative to Experiment 1 (which included two; see Table 1). Third, rats show large but stable individual differences in their proclivities to produce these vocalizations (12). None of these issues (i.e., transiency, shifting baselines, or individual differences) is inconsistent with the hypothesis that 50-kHz vocalizations index positive affective states, but they do complicate measurement attempts. In our hands, 50-kHz vocalizations show impressive sensitivity to hedonic associations, provided that incentive stimuli are contrasted with nonincentive stimuli in within-subject ratio measurements and with properly controlled comparison groups.

Although some questions exist regarding the specificity of these vocalizations to rewarding contexts, a converging body of evidence supports this association. One ambiguity arises from the observation that, in addition to making these vocalizations prior to rewarding social interactions [i.e., play (12); sex (2)], male rats also make 50-kHz vocalizations prior to fighting (13). Typically, they are observed at the beginning of aggressive episodes, but almost never during sustained defeat (21). Nevertheless, three pieces of evidence support the hypothesis that short 50-kHz USVs mark not only a state of high arousal, but also one of positive expectations. First, 50-kHz USVs are correlated with other preference measures, such as spending time in environments associated with prior social or pharmacological reward (12). Second, rats make these vocalizations in anticipation of rewarding brain stimulation (6). Third, aversive yet arousing stimuli such as bright light, open spaces, and unexpected termination of rewarding brain stimu-

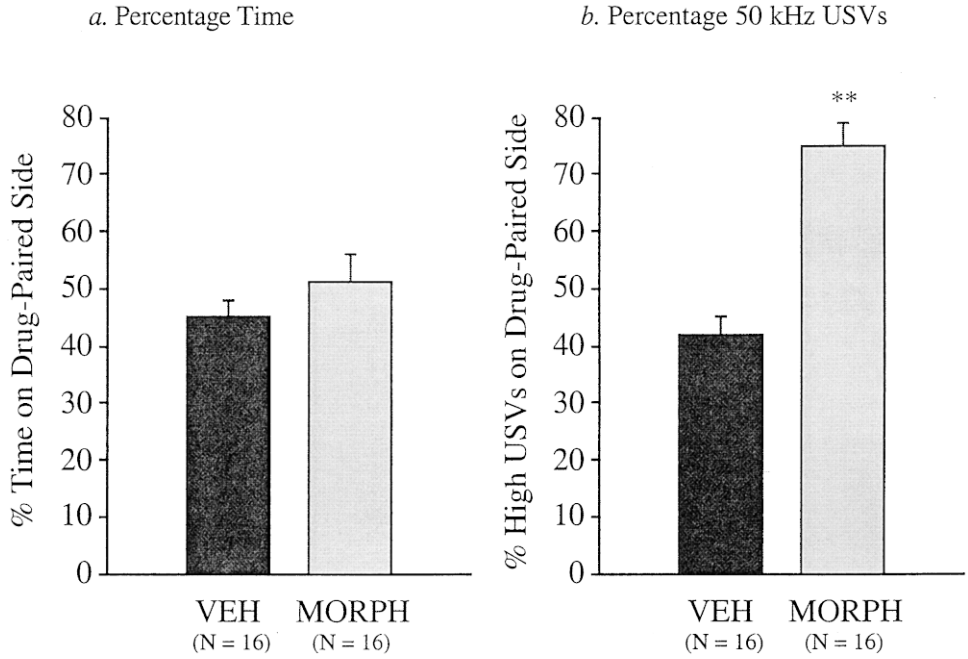

FIG. 2. Percentage time and 50-kHz USVs in MORPH-paired chamber (error bars = SEM, $*p < 0.05$).

lation tend to eradicate 50-kHz vocalizations, while simultaneously instigating 22-kHz vocalizations (6,12).

Even if 50-kHz USVs do mark a positive affective state, many questions regarding generalizability remain to be addressed. For instance, we utilized isolate-reared rats as subjects. Other investigators have reported that isolates tend to show greater sensitivity to pharmacological agents with abuse potential (10). On the other hand, socially housed animals may spontaneously vocalize more than isolates (25), so it is not clear how rearing might affect 50-kHz USVs in our paradigm. Also, although we selected the minimal doses of AMPH and MORPH necessary to elicit conditioned place preference, the effects of other doses on USVs requires characterization. Finally, regardless of their acute pharmacological effects, both AMPH and MORPH have been hypothesized to induce place preference via eventual modification of central dopaminergic pathways (18,19). Other compounds that have abuse potential in humans but not any obvious dopaminergic actions might warrant investigation with the present paradigm.

In any event, these findings address some criticisms that have been leveled at the conditioned place-preference paradigm, such as the possibility that amnesia rather than hedonic associations causes place preference (16). Our observations particularly allay some of these concerns with respect to MORPH (17). Although rats made more 50-kHz USVs in a place where they had received MORPH in Experiment 2, they tend to make fewer 50-kHz USVs in novel environments (12). Thus, if rats could not remember the site where they had received MORPH, one would expect them to vocalize less there rather than more. This clarification suggests that 50kHz USVs may eventually provide a sensitive measure of reward conditioning that complements traditional place-preference designs.

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