Nucleus Accumbens Amphetamine Microinjections Unconditionally Elicit 50-kHz Ultrasonic Vocalizations in Rats

Jeffrey Burgdorf and Brian Knutson
National Institutes of Health

Jaak Panksepp
Bowling Green State University

Satoshi Ikemoto
National Institutes of Health

The authors have hypothesized that, in adult rats, 50-kHz ultrasonic vocalizations (USVs) index a state characterized by high arousal and expectations of reward. This study was conducted to investigate whether dopamine agonism of the nucleus accumbens (NAcc) could evoke such an appetitive state, by examining the effects of NAcc amphetamine (AMPH) microinjections on USVs. Intra-NAcc AMPH injections (0.3, 1.0, 3.0, 10.0 μg unilaterally) produced robust, dose-dependent increases in 50-kHz USVs, which could not be accounted for by concomitant increases in locomotor activity (LA). However, AMPH injections into dorsal control caudate putamen sites produced a modest, dose-dependent increase in LA without significant increases in 50-kHz USVs. These findings indicate that NAcc AMPH microinjections selectively evoke 50-kHz USVs in rats, supporting the notion that dopamine elevations in the NAcc may unconditionally elicit a state of reward anticipation.

Presentation of cues that predict rewards can elicit spontaneous appetitive behaviors in rats, including forward locomotion and sniffing (Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999; Pavlov, 1927). But in addition to these visible skeletal motor changes, reward cues can also modulate visceral systems, as evidenced by changes in heart rate and blood pressure (Hunt & Campbell, 1997). Additionally, emerging evidence suggests that presentation of cues for various types of rewards can also increase the expression of short (<300 ms) 50-kHz ultrasonic vocalizations (USVs) in rats. For example, male rats emit 50-kHz USVs when presented with cues that predict access to a sexually receptive female (Barfield, Auerbach, Geyer, & Mcintosh, 1979); juvenile rats emit 50-kHz USVs when presented with cues that predict the opportunity to play with a conspecífic (Knutson, Burgdorf, & Panksepp, 1998); and adult rats emit 50-kHz USVs when presented with cues that predict (a) rewarding brain stimulation, (b) food delivery (Burgdorf, Knutson, & Panksepp, 2000), and (c) drugs of abuse (Knutson, Burgdorf, & Panksepp, 1999).

Because the presentation of reward cues can elicit 50-kHz USVs in rats, Knutson et al. (1999) hypothesized that these vocalizations may index a positive affective state characterized both by high arousal and by anticipation of reward. It appears that 50-kHz USVs selectively index this positive affective state because the presentation of cues associated with aversive stimuli (e.g., footshock, predation) instead decreases their expression (Blanchard, Blanchard, Agullana, & Weiss, 1991; Burgdorf et al., 2000; Burgdorf, Knutson, Panksepp, & Shippenberg, 2001). Presentation of aversive cues does, however, evoke a different type of USV (Antoniadis & McDonald, 1999; Burgdorf et al., 2001), which can be distinguished on the basis of its longer length (>300 ms) and lower frequency (i.e., 20-kHz USVs; Brudzynski & Ociepa, 1992). Accordingly, other theorists have hypothesized that 20-kHz USVs index a negative affective state characterized by high levels of arousal but with expectations of punishment (Miczek, Weerts, Vivian, & Barros, 1995; Tonoue, Ashida, Makino, & Hata, 1986).

Considerable evidence has indicated that dopamine (DA) projections from the ventral tegmental area of the midbrain to the nucleus accumbens (NAcc) of the ventral striatum may play a central role in modulating affective states involving both high levels of arousal and anticipation of reward. These appetitive states can be distinguished from consummatory states, which occur after reward delivery, and involve distinct neural mechanisms (Berridge & Robinson, 1998; Ikemoto & Panksepp, 1999; Panksepp, 1998; Wise, 1998). DA modulation of the NAcc particularly appears to induce an appetitive state in rats because rats will vigorously self-administer DA agonists into this region (and into the NAcc shell in particular) but not into other nearby DA terminal areas, such as the caudate putamen (CPU; Carlezon, Devine, & Wise, 1995; Hoebel et al., 1983; Ikemoto, Glazier, Murphy, & McBride, 1997). Microinjection of DA agonists into the NAcc also power-
fully elicits place-preference behavior (McBride, Murphy, & Ikemoto, 1999). However, stressors can also increase DA release in the NAcc, and NAcc DA depletion impairs active avoidance as well as approach behaviors (Salomone, Cousins, & Snyder, 1997). These findings suggest that NAcc DA may generally modulate both aversive and appetitive states rather than selectively modulating appetitive states, although avoidance behavior usually includes both an appetitive component related to anticipated escape from punishment as well as an aversive component related to anticipated punishment (Ikemoto & Panksepp, 1999).

In this study, we reasoned that if increases in NAcc DA induce an appetitive state in rats, then a microinjection of the mixed DA agonist amphetamine (AMPH) into the NAcc should unconditionally and dose-dependently elicit 50-kHz USVs. On the other hand, if an AMPH microinjection into the NAcc amplifies general arousal rather than selectively intensifying appetitive states, then this manipulation should increase both 50-kHz USVs and 20-kHz USVs.

Method

Subjects

Nineteen male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing 250 to 300 g, were housed 2 or 3 per cage in a temperature- and humidity-controlled colony room prior to surgery, and were thereafter individually housed. Rats were maintained on a 12:12-hr light–dark cycle (lights on at 9:00 p.m.) and received ad libitum access to laboratory rat chow (Ralston-Purina, St. Louis, MO) and water throughout the experiment except in test chambers. Rat facilities were accredited by the American Association for the Accreditation of Laboratory Animal Care, and the experiment was approved in advance by the Institutional Care and Use Committee of the Divisions of Intramural Research, National Institute on Drug Abuse of the National Institutes of Health.

Surgery

Thirteen rats were randomly assigned to receive NAcc cannula implants, and the remaining 6 received implants in the CPu. Rats were first anesthetized with an intraperitoneal injection of Equithesin (3.2 mg/kg). Permanent bilateral guide cannulas were then stereotaxically implanted in either the NAcc (1.8 mm anterior, 3.2 mm lateral, 6.6 mm ventral to bregma) or the CPu (1.8 mm anterior, 3.2 mm lateral, 4.6 mm ventral to bregma). All coordinates were taken from flat brain orientation, and cannulas were angled 16° away from midline in order to avoid the lateral ventricles. Injection cannulas extended 1.0 mm beyond the end of the guide cannulas.

Drug Administration

Either AMPH (0.3, 1.0, 3.0, 10.0 µg/0.5 µl; National Institute on Drug Abuse, Baltimore, MD) or vehicle (0.9% [vol/vol] saline) were administered via injection cannula (31 gauge) inserted into the implanted guide cannula (24 gauge). Injections (0.5 µl volume) were made unilaterally (on the left) over a period of 60 s. After injection was completed, we left the injection cannula in place for an additional 30 s before removing it. Subjects were then placed into the locomotor testing chamber for behavioral testing.

Behavioral Testing

Rats were habituated to the locomotor chamber for 3-consecutive days before behavioral testing began. Each subject received injections of all four doses of AMPH and one vehicle injection in a counterbalanced order across rats and test days, with at least 48 hr separating each testing session. During each break between testing sessions, rats received one rehabilitation session by being placed in the testing chamber for 30 min to minimize potential place-conditioning effects. Immediately following injections, rats were individually placed into a sound- and light-attenuated locomotor chamber (40 × 40 × 30 cm; Digiscan Animal Activity Monitoring System, AccuScan Instruments, Columbus, OH). Locomotor distance (total distance in centimeters traveled by the rat) was automatically recorded by a personal-computer monitoring system for a 30-min session following each injection.

During this time, a rater recorded 50- and 20-kHz USVs by listening to a Pettersen D980 ultrasonic detector (Uppsala, Sweden). One channel was tuned so that all USVs could be heard (the broadband channel: 10–20 kHz), whereas the other channel was tuned to the 20-kHz range (i.e., 20 ± 6 kHz). We determined that USVs that occurred in both channels were 20-kHz USVs, and that the USVs heard only in the broadband channel were 50-kHz USVs (Burgdorf et al., 2001). In addition to the channel of occurrence, raters also used distinct temporal characteristics to discriminate the two USV types (i.e., 50 kHz < −500 ms whereas 20 kHz > −500 ms; Brudzynski, Bihari, Ociepa, & Fu, 1993). High interrater reliability (Spearman’s rs = .90) has been established for this method of coding USVs in prior studies (Burgdorf et al., 2000; Knutson et al., 1998).

Histology

After completing the experiment, rats received a 0.5-µl intracranial injection of Evans Blue. Their brains were then removed and sectioned in 60-µM slices on a cryostat. Brain tissue was slide-mounted and coverslipped for microscopic visualization. Microinjection placements were localized either in the NAcc (n = 13 total: core n = 9, shell n = 4) or in the CPu (n = 6 total), as defined by Paxinos and Watson (1997; see Figure 1).

Results

USVs

Because rats made no 20-kHz USVs during behavioral testing, these vocalizations are not discussed further. We analyzed 50-kHz USVs with a 2 (microinjection placement: NAcc vs. CPu) × 5 (microinjection dose–within: vehicle [VEH] vs. 0.3, 1.0, 3.0, or 10.0 µg/0.5 µl AMPH) factor repeated measures analysis of variance (ANOVA). To ensure that changes in USVs were not due to changes in locomotor activity (LA), an identical second analysis was performed with changes in LA entered as a covariate.

The first analysis of 50-kHz USVs revealed significant main effects of placement, F(1, 17) = 10.74, p < .005, and dose, F(4, 68) = 15.86, p < .001, qualified by an interaction of placement and dose, F(4, 68) = 5.64, p < .001. Tukey’s honestly significant difference (HSD) post hoc pairwise comparisons indicated that each of the highest three doses of AMPH elicited significantly more 50-kHz USVs than VEH for the NAcc placement group (p < .001), but that none of the AMPH doses elicited significantly more USVs than the VEH for the CPu placement group (see Figure 2A).

When changes in LA were included in this analysis as a covariate, results revealed a main effect of placement, F(1, 16) = 5.14, p < .05, qualified by an interaction of placement and dose, F(4, 64) = 3.29, p < .05. Tukey’s HSD tests for this analysis indicated that all AMPH doses elicited significantly more 50-kHz USVs than the VEH dose for the NAcc placement group only, but not for the CPu placement group. Thus, changes in our locomotor measure could not statistically account for the presence of an AMPH
Effect of NAcc Microinjection Placement

Retrospective visualization of the histology revealed that not all microinjection placements fell within the same subareas of the NAcc. Specifically, 4 rats received microinjections in the shell of the NAcc, and the remaining 9 received microinjections in the core of the NAcc (see Figure 1). This difference permitted us to conduct a subsequent preliminary analysis to determine whether microinjection placement within the NAcc group influenced the ability of AMPH to elicit 50-kHz USVs and LA. Thus, for the NAcc group only, a 2 (placement) × 5 (dose—within) repeated measures ANOVA on 50-kHz USVs covarying for LA revealed a main effect of placement, $F(1, 10) = 14.18, p < .005$, and a main effect of dose, $F(4, 40) = 8.08, p < .001$, qualified by an interaction of placement and dose, $F(4, 40) = 7.35, p < .001$. Tukey’s HSD tests revealed that all doses of AMPH, but not VEH, elicited significantly more 50-kHz USVs for the shell placement group than for

---

**Figure 1.** Microinjection sites for nucleus accumbens ($n = 13$; filled circles) and caudate putamen ($n = 6$; open circles) placement groups. From *The Rat Brain in Stereotaxic Coordinates* (3rd ed., Figures 10–13) by G. Paxinos and C. Watson, 1997, San Diego, CA: Academic Press. Copyright 1997 by Academic Press. Adapted with permission.

dose-related increase in 50-kHz USVs as seen in the NAcc placement group.

**LA**

We analyzed LA similarly, with a 2 (placement) × 5 (dose—within) repeated measures ANOVA. This analysis revealed a significant main effect of placement, $F(1, 17) = 6.23, p < .05$, and dose, $F(4, 68) = 21.24, p < .001$, qualified by an interaction of placement and dose, $F(4, 68) = 3.43, p < .05$. Tukey’s HSD tests again revealed that the highest three doses of AMPH elicited more LA than VEH in the NAcc placement group, but that LA did not differ across doses for the CPU placement group (see Figure 2B).

However, when 50-kHz USVs were included in the same analysis as a covariate, the analysis yielded a significant main effect of dose, $F(1, 16) = 6.69, p < .005$, but no significant interaction term, $F(4, 64) = 1.74, ns$. Tukey’s HSD tests indicated that for both NAcc and CPU placement groups, the three highest doses of AMPH produced more LA than VEH did. Thus, changes in 50-kHz USVs could statistically account for the interactive effect of microinjection placement and dose on LA.

---

**Figure 2.** Dose–response plot of mean (±SEM) 50-kHz ultrasonic vocalization (USV) frequency (A) and locomotor activity (B) for nucleus accumbens (NAcc) shell ($n = 4$), NAcc core ($n = 9$), and caudate putamen (CPU; $n = 6$) placement groups. Graph insets compare the CPU group to a collapsed NAcc group ($n = 13$) for both 50-kHz USV frequency and locomotor activity.
the core placement group at equivalent dose levels (see Figure 2A). However, when LA was subjected to a similar analysis covarying for 50-kHz USVs, a significant main effect of dose was observed, \( F(4, 40) = 2.99, p < .05 \), but no significant interaction was obtained, once again revealing a dissociation between LA and 50-kHz USVs.

**Discussion**

These results demonstrate that microinjection of AMPH into the NAcc unconditionally and dose-dependently elicits 50-kHz USVs but not 20-kHz USVs. This dose-dependent increase in 50-kHz USVs did not occur in dorsal control rats that received AMPH injections into the CPu. Furthermore, subsequent analyses suggest that microinjection of AMPH into the shell of the NAcc elicits more 50-kHz USVs but not more LA than microinjection of AMPH into the core of the NAcc. Finally, in all instances, covariance analyses indicate that changes in LA cannot statistically account for changes in 50-kHz USVs.

It has been suggested that 50-kHz USVs are generated as a byproduct of LA. According to this account, forepaw contact with the ground compresses the diaphragm, which then propels air over the larynx, and so produces vocalizations (Blumberg, 1992). However, this hypothesis cannot account for several aspects of our findings. First, although NAcc shell placements elicited more 50-kHz USVs than NAcc core placements, NAcc shell placements did not elicit more LA. Second, although NAcc placements elicited both more 50-kHz USVs and LA than CPu placements, covariance of changes in LA could not statistically account for observed increases in 50-kHz USVs. These observed dissociations between the expression of 50-kHz USVs and LA concur with dissociations we have previously observed between these two types of behavior. Specifically, we have observed increases, decreases, and no change in LA with increases in 50-kHz USVs (Burgdorf et al., 2000; Knutson et al., 1998; Knutson, 1989; Panksepp & Burgdorf, 2000). However, in this study, we found that an unsignalled unconditional stimulus can also powerfully evoke these vocalizations. One possible interpretation of this phenomenon is that AMPH microinfusion into the NAcc directly induces a positive and aroused state, which typically occurs during anticipation of reward. Such a state would correspond more closely to traditional notions of appetitive motivation rather than consummatory motivation (Sherrington, 1906).

This is the first study to demonstrate that intracranial injections of AMPH can elicit 50-kHz USVs. Interestingly, stimulation of other brain regions can also elicit USVs in rats, but the sites that support 20-kHz USVs are anatomically or neurochemically distinct from those that support 50-kHz USVs. For example, injections of glutamate into the preoptic area trigger 50-kHz USVs but not 20-kHz USVs, whereas injections of cholinergic agonists into the preoptic area elicit 20-kHz USVs but not 50-kHz USVs (Fu & Brudzynski, 1994). Sites where electrical stimulation elicits 20-kHz USVs also include areas typically implicated in nociception such as the medial hypothalamus, medial thalamus, and periaqueductal gray (Yajima, Hayashi, & Yoshii, 1980, 1981). On the other hand, brain sites in which rats will self-administer DA agonists may provide candidate loci for eliciting 50-kHz USVs. This prediction fits with our current observations that AMPH microinjection into the NAcc elicits 50-kHz USVs more potently than AMPH microinjection into the CPu and that AMPH microinjection into the NAcc shell elicits 50-kHz USVs more potently than AMPH microinjection into the NAcc core (McBride et al., 1999). To more thoroughly evaluate this hypothesis, other brain sites where rats will self-administer DA agonists besides the NAcc also need to be evaluated, such as the medial prefrontal cortex.

Even though AMPH affects other biogenic amine systems in addition to DA, it is likely that AMPH elicited 50-kHz USVs by increasing DA release in the NAcc because administration of glutamate to the ventral tegmental area (which stimulates NAcc dopamine release) also unconditionally elicits 50-kHz USVs (Burgdorf, 2000). AMPH blocks dopamine reuptake in the NAcc, yielding enhanced DA agonism of both D1 and D2 receptors. Because a combination of D1- and D2-type receptor agonists in the NAcc shell supports self-administration behavior more potently than administration of either agonist in isolation (Ikemoto et al., 1997), we predict that simultaneous stimulation of both D1- and D2-type receptors should elicit more vigorous 50-kHz USVs than agonism of either receptor type in isolation. However, to conclusively establish that NAcc microinjections of AMPH increase 50-kHz USVs by means of a dopaminergic mechanism, researchers would need to demonstrate that this effect could be blocked by dopaminergic antagonists but not by noradrenergic antagonists.

In conclusion, microinjection of AMPH into the NAcc unconditionally and dose dependently elicits 50-kHz USVs but not 20-kHz USVs in adult rats. This effect was more pronounced for NAcc shell injections than for NAcc core injections. Covariance analyses suggest that changes in LA could not account for increases in 50-kHz USVs. These findings are consistent with the hypothesis that DA elevations in the NAcc may induce an affective state characterized by high arousal and positive expectations in rats—a state that may also be powerfully recruited by the anticipation of natural rewards (Ikemoto & Panksepp, 1999; Panksepp, 1998).

**References**


Received August 8, 2000
Revision received January 22, 2001
Accepted February 8, 2001