



## Research report

# Ultrasonic vocalizations in rats anticipating circadian feeding schedules

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## HIGHLIGHTS

- Activity and ultrasonic vocalizations (USVs) were recorded for 2 h or 24 h in rats fed ad libitum or 2 h per day in the light period.
- Food restricted rats exhibited a daily rhythm of food anticipatory activity.
- Food anticipation was accompanied by FM and flat ~50 kHz USVs.
- Flat ~50 kHz USVs were more dependent on the presence of food.
- An audible tone prior to mealtime modulated anticipatory locomotor activity but did not evoke ~50 kHz USVs.

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## ABSTRACT

Rats readily learn to anticipate a reward signaled by an external stimulus. Anticipatory behaviors evoked by conditioned stimuli include 50 kHz ultrasonic vocalizations (USVs), a proposed behavioral correlate of positive affect and activation of midbrain dopamine pathways. Rats can also anticipate a reward, such as food, provided once daily, without external cueing. Anticipation of a daily reward exhibits formal properties of a circadian rhythm. The neural circuits that regulate the timing and amplitude of these rhythms remain an open question, but evidence suggests a role for dopamine. To gain further insight into the neural and affective correlates of circadian food anticipatory rhythms, we made 2 h and 24 h USV recordings in rats fed 2 h/day in the light period, a procedure that induces robust anticipation 2–3 h before mealtime. Potential interactions between internal and external time cues in USV production were evaluated by inclusion of a 3 kHz tone 15 min before mealtime. Prior to scheduled feeding, spontaneous 50 kHz USVs were rare during the light period. During scheduled feeding, flat and frequency modulated (FM) 50 kHz USVs occurred prior to and during mealtime. FM USVs were more closely related to anticipation, while flat USVs were more dependent on food access. USVs also occurred during spontaneous waking at other times of day. The tone did not evoke USVs but did modulate activity. Behavioral anticipation of a daily meal is accompanied by USVs consistent with a positive affective state and elevated dopamine transmission.

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## 1. Introduction

Rats emit distinct ultrasonic vocalizations (USVs) that occur in different behavioral contexts and are believed to signal different affective states [1–4]. So-called low frequency (Low) USVs in the 20–30 kHz range are thought to reflect negative affective states, associated with aversive stimuli and activation of mesolimbic

cholinergic neural pathways [5–9]. These may function as alarm calls to alert other rats to sources of distress, or warnings to keep away (e.g., during a post-copulatory pause) [10,11]. Conversely, high frequency (High) USVs in the 30–90 kHz range are thought to reflect positive affective states, associated with anticipation or acquisition of appetitive stimuli (e.g., addictive drugs, brain stimulation reward, social interactions) and activation of mesolimbic dopaminergic neural pathways [12–18]. High USVs can also be elicited by conditioned stimuli (e.g., a tone or light) that predict reward [15,19], and USV playback can induce phasic dopamine release and approach behavior [20]. High USVs can be frequency modulated (FM) or flat (constant frequency). The FM subtype appears to be more strongly associated with reward stimuli and

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dopamine signaling, while the flat subtype has been associated more strongly with social conditions ([3,16,21]. One study that used cluster analysis to identify behavioral correlates of high frequency USVs found that FM calls associated with movement and flat calls with feeding [22]. Although dissociations between High USVs, positive affect and reward stimuli have been noted [23–25], a hypothesis that these calls, particularly the FM subtype, ‘index motivation during reward anticipation’ [15] remains tenable.

The association of FM High USVs with reward anticipation, reward acquisition, and activation of dopaminergic pathways prompted us to ask whether High USVs also occur in food restricted rats anticipating a daily meal. Restricted feeding schedules in which food is provided once daily in the middle of the light period induce a daily rhythm of food anticipatory locomotor activity in nocturnal rats and mice. Formal properties of this rhythm are consistent with control by food-entrainable circadian oscillators (FEOs) [27–29]. These properties include persistence of food anticipatory rhythms for several cycles during total food deprivation, and failure of anticipatory rhythms to emerge if the periodicity of the feeding schedule falls outside of a circadian range [30–33]. Many questions remain about the mechanisms by which FEOs regulate food anticipatory behavior, including the location of these oscillators in the brain or body. Lesion studies have ruled out the suprachiasmatic nucleus (SCN), a master circadian pacemaker for light–dark (LD) entrained rhythms, as well as a number of other hypothalamic and forebrain structures that express circadian clock genes or that participate in regulation of feeding and metabolism [28,34,35]. A role for dopaminergic signaling in the timing and magnitude of circadian food anticipatory rhythms is suggested by several findings. Food anticipatory activity can be shifted by dopamine D2 receptor agonists in rats [36] and anticipatory activity can be induced by a daily injection of D1 and D2 receptor agonists in rats [37] and D1 receptors in mice [38]. Conversely, food anticipatory activity is absent in dopamine deficient mice and markedly attenuated by D1 receptor knockout [38] and D1 receptor antagonists [39]. Notably, D1 receptor antagonists also suppress High USVs elicited by conditioned stimuli that predict a palatable food reward [15]. These results suggest that dopamine signaling and activation of D1 receptors plays a critical role in the expression of food anticipatory locomotor activity and High USVs. This leads to a prediction that rats will emit High USVs when anticipating a daily opportunity to feed.

The mechanism by which FEOs interact with behavioral output systems is also uncertain [28]. FEOs may drive a daily rest-activity cycle, analogous to how the SCN pacemaker is understood to alternately promote wake and sleep in synchrony with the solar day [40]. According to this model, anticipation of a daily meal need not have a cognitive or affective component; rats may become active prior to a regular feeding time as a consequence of the phase of entrainment of a circadian oscillator, without cognitive or affective anticipation of imminent food delivery. Alternatively, FEOs may provide a continuous readout of circadian phase, by which time of day can be discriminated and encoded as a contextual cue in memories of prior feeding events (e.g., [41,42]). According to this model, locomotor activity expressed prior to a daily meal would be considered an expression of circadian phase-dependent incentive motivation, and likely would be accompanied by high frequency USVs signaling expectation of food.

To gain further insight into the processes and neural substrates responsible for generating anticipatory activity rhythms synchronized with a daily feeding opportunity, we recorded USVs in rats prior to a scheduled daytime meal and for 24 h intervals during restricted feeding and ad libitum food access. Continuous 24 h recordings of spontaneous USVs in undisturbed, single housed rats have not previously been reported, to our knowledge. We included in the protocol an auditory tone as a potential conditioned stimulus for mealtime, which could then be presented at other times of

day. We observed that rats do emit USVs in the 30–70 kHz range prior to a daily meal, although these calls did not come under control of the auditory stimulus, despite evidence for a conditioned effect of the tone on locomotor activity. We also found a distinction between FM and Flat high frequency USVs; FM calls increased at the expected mealtime and decreased when food was provided, while Flat calls increased at the expected mealtime but decreased if food was withheld.

## 2. Materials and methods

### 2.1. Subjects and apparatus

Sixteen young adult male Sprague–Dawley rats (Charles River, Montreal, QB), weighing  $313 \pm 16$  g at the start of the study, were individually housed in polypropylene cages ( $45 \times 24 \times 20$  cm) with corncob bedding. Frizzy-paper and a translucent toy cube were provided for enrichment. A 12:12 light–dark (LD) cycle was maintained. Behavioral activity was measured by motion sensors (Quorum RR-150) mounted above the cage. Sensors were monitored continuously using the ClockLab data acquisition system (Actimetrics, IL, USA). The motion sensors can detect any movement, but most counts represent forward locomotion and rearing associated with inspection of the food hopper. USVs were recorded using a BAT AR125 ultrasonic receiver (frequency range 1–125 kHz) and the SPECTR data acquisition system (Binary Acoustics Technology; binaryacoustictech.com). The USV receiver was placed at a 45-degree angle facing the inside of the cage, at a distance of 25 cm from the cage bottom. All animal work was conducted according to guidelines established by the Canadian Council on Animal Care and was approved by the University Animal Care Committee at Simon Fraser University.

### 2.2. Procedure

From age 18–22 weeks the rats were handled daily, with food available ad libitum (Purina rat chow no. 5001). USV recordings were made during ad libitum food access prior to restricted feeding, although these baseline recordings were not used for reasons discussed below. All rats were then subjected to a sequence of four conditions (Fig. 1). In Condition 1 (days 1–33), food was restricted to a 2-h daily meal beginning 3-h after lights-on (Zeitgeber Time 3, where, by convention, ZT0 is lights-on). The daily mealtime was signaled by a 3 kHz tone ( $\sim 73$  dB), beginning 15 min before mealtime (ZT2.75) and ending 5 min after food delivery. USV recordings were made from ZT2–4 from one rat per day. The ultrasonic receiver was moved to a new cage each day at ZT4. Each rat was recorded twice, at 16-day intervals. The objective of this condition was to determine whether USVs would occur prior to a daily meal, either spontaneously or by conditioning to an auditory cue predicting mealtime. A second objective was to identify rats that consistently emitted USVs during food restriction.

In Condition 2, the 3 kHz tone was sounded twice daily for 20 min, once at ZT2.75 and again at ZT10 (2 h before lights-off), for 29 days (days 34–62 of restricted feeding). USV recordings were made from ZT2–4 and again from ZT9–10.5, beginning 60 min prior to the second tone. The objectives of this condition were to determine whether the tone would elicit conditioned USVs and/or locomotor activity at a time of day not previously associated with feeding, and to determine whether the rats might begin to anticipate this second auditory tone, if the tone had acquired secondary reward properties. Eight rats with the highest levels of USVs in Condition 1 were recorded twice, at  $\sim 16$  day intervals. During the second round of USV recordings, the rat to be recorded on that day was not fed, to determine whether USVs might be elicited by the

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