Published in final edited form as:

Physiol Behav. 2013 March 15; 0: 56–60. doi:10.1016/j.physbeh.2013.02.011.

Influence of estrous and circadian cycles on calcium intake of the rat

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Abstract

The food, water and sodium intake of laboratory rats fluctuates over the circadian and estrous cycles. Blood calcium and calcium-regulating hormones also wax and wane in response to these cycles, raising the possibility that the same might be true of calcium intake. To investigate this, we monitored the fluid intakes of female Long-Evans rats given a choice between water and 10 mM CaCl_2 solution for two consecutive estrous cycles. We found that calcium solution intake changed over the circadian cycle in a similar manner to water intake; the preference scores for CaCl_2 solution remained stable. We did not detect any changes in calcium solution intake or preference scores during the estrous cycle despite a decrease in fluid intake at estrus. Thus, fluctuations in intake of calcium solution during the circadian cycle appear to be nonspecific and probably the result of changes in fluid balance. Estrous changes either do not influence calcium intake or their effects are masked by other factors, resulting in stable levels of calcium intake.

Keywords

drinking patterns; diurnal; estrogen; progesterone; TSE Phenomaster

1. Introduction

In female laboratory rats, ingestive behavior fluctuates over the estrous cycle with a pronounced decrease during the estrus stage—an event attributed to the delayed action of estrogens peaking during the proestrus stage [1–4]. Many aspects of calcium metabolism including plasma calcium, intestinal calcium absorption and calcitonin levels also fluctuate over the estrous cycle. For example, in the rat, plasma calcium levels are significantly higher during the morning of proestrus than during the diestrus stages, and intestinal calcium absorption is highest during the estrus stage when calcitonin levels are low [5,6]. The increase in calcium absorption during estrus can be blunted by ovariectomy, and estradiol replacement increases calcium absorption in ovariectomized females [5,7,8]. Administration of estradiol also increases calcium intake of calcium-replete female rabbits [9,10] and gonadectomized male chickens fed low-calcium diet [11]. Other reproductive hormones also increase CaCl_2 intake [10,12,13]. Thus, calcium metabolism fluctuates over the estrus cycle, and manipulations of reproductive status influence calcium intake, but whether calcium

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intake is influenced by the naturally occurring fluctuations in reproductive hormones occurring over the estrus cycle is unknown

Superimposed on the estrous cycle is a circadian cycle. Typically, 70–85% of a rat's daily water and food consumption occurs during the dark phase [14–17]. Plasma levels of calcium and calciotropic hormones also change over the circadian cycle [15,18]. It is therefore logical to suppose that, in addition to estrous fluctuations, circadian fluctuations influence calcium intake. However, to our knowledge, whether calcium intake fluctuates during the circadian cycle has not been assessed.

The aims of this study were thus to characterize the relationship between estrous cycle, circadian rhythms and voluntary calcium intake. To do so, we measured minute-by-minute intakes of 10 mM CaCl_2 solution in two-bottle choice tests over the course of two estrous cycles in female laboratory rats under *ad libitum* conditions.

2. Methods

The experiment protocol was approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee.

2.1. Subjects

The experiment was conducted in two batches involving 36 rats in total. Female Long Evans rats were obtained from Charles River (Raleigh, NC) at the age of 70 days. Each rat was initially housed individually in a stainless steel wire mesh hanging cage ($19.5 \times 17.5 \times 24$ cm). AIN-76A purified pelleted diet (#100000, Dyets, Bethlehem, PA) containing 130 mmol calcium/kg diet and deionized water were provided *ad libitum*. The vivarium was maintained at 23°C on a 14:10 h light/dark cycle with lights off at 20:00 and on at 06:00. Testing began when the rats were 91–104 days old.

2.2. Choice of the test solution

We used 10 mM CaCl_2 solution to examine fluctuations in calcium intake. In a previous strain survey using 48-h two-bottle choice tests, female Long Evans rats showed ~40% preference scores for 10 mM CaCl_2 relative to water; preference scores were much lower at higher concentrations [19]. We reasoned that the 10 mM concentration was distinguishable from water by the rats but it also had the potential to allow increases and decreases in intake to occur without undue concerns that basement or ceiling effects might limit responses.

2.3. Determination of estrous cycle phase

Each rat's estrous stage was determined based on vaginal cytology. Vaginal smears were taken daily throughout the experiment, at ~09:00. The stage of the cycle determined at the beginning of the light period was assigned to that light period and to the preceding dark period (i.e. starting from 20:00 of the previous calendar day). Only rats having two consecutive 4-day estrous cycles before testing began were included in the study.

2.4. Behavioral testing

A total of 28 rats demonstrated consistent 4-day cycles and were moved into cages designed to record fluid intakes (Phenomaster, TSE Systems, Bad Homburg, Germany). The Phenomaster apparatus allows continuous recording of water intake from containers that are suspended from load cells (weighing sensors; ± 0.01 g accuracy). Each rat was housed in a $20 \times 37 \times 15$ cm plastic “tub”-style cage with a stainless steel wire lid and aspen shavings as bedding material. In the configuration used for this experiment, 100-mL drinking bottles were hung from the weighing sensors so that each spout was ~6 cm from the floor and 2.5

cm from the nearest side wall; the distance between the two spouts was ~16 cm. Pelleted AIN-76A food was provided in a hanging basket located between the drinking bottles at ~4 cm distant from each bottle.

Each drinking bottle was made of glass and had an aluminum cap that protected the plastic stopper from being gnawed. Drinking spouts were made of stainless steel. To prevent spillage, each drinking spout had a stainless steel pin that protruded ~2 mm from the spout opening so that in order to drink a rat had to push the pin to the side with its tongue. Most rats learned to drink from these bottles within a few hours. Testing started at least 3 days after all rats exhibited steady drinking from both bottles.

First, the intakes from two bottles, each containing deionized water, were recorded for one 4-day estrous cycle. Then, the rats were given a choice between deionized water and 10 mM CaCl_2 solution (Sigma-Aldrich, St. Louis, MO; dissolved in deionized water). The solution was always presented on the right. Rats were tested with the solution for 9 days (two consecutive cycles and one day). Water, solution and food containers were refilled at ~09:00 when vaginal smears were taken.

2.5. Data analysis

Intakes were converted from grams to milliliters with the assumption that the water and CaCl_2 solution each had a density of 1 mL/g. A drinking bout was defined as any intake from a bottle that was detected (i.e., >10 mg) and separated from the previous bout by at least 5 min. Bout size was determined by dividing water or solution intake by the number of bouts during each time period. Occasional intakes of >2 mL per minute were recorded and these were treated as a spill and discarded from analysis.

Only data from rats that had a consistent 4-day estrous cycle throughout all tests were included in analyses. Five rats dislodged a drinking bottle from its sensor and so their data were excluded from the analyses for the respective test. This left 20 and 17 rats in the deionized water and 10 mM CaCl_2 tests, respectively. Recordings made during the first day of the 9-day choice test were excluded from data analysis so as to minimize the influence of novelty on consumption patterns.

The rats were not cycling in synchrony. Consequently, testing was organized so that ~25% of the rats were presented with the solution on the first day of the estrous cycle (i.e. during diestrus 1), ~25% on the second day (diestrus 2), ~25% on the third day (proestrus) and ~25% on the fourth day (estrus). This was done to exclude the influence of the duration of presentation of the solution on the observed intake patterns over the estrous cycle.

The light and dark periods of the circadian cycle differed in duration (14 h and 10 h respectively) so to analyze circadian drinking patterns, we blocked data into two equal 5-h (300 min) dark period intervals and three equal 4.67-h (280-min) light period intervals. This gave the following periods: 20:00 – 1:00 (Dark 1), 01:00 – 06:00 (Dark 2), 06:00 – 10:40 (Light 1), 10:40 – 15:20 (Light 2), and 15:20 – 20:00 (Light 3).

Intakes from each bottle were analyzed separately using repeated measures analyses of variance (ANOVAs). Each dependent variable (water intake, CaCl_2 intake, total fluid intake, CaCl_2 preference scores, water bout size, CaCl_2 bout size, water bout number and CaCl_2 bout number) was analyzed using repeated-measures ANOVAs with factors of Estrous Cycle Number (i.e., the first or second of the two consecutive cycles recorded), Time of Day (5 time periods specified above) and Stage of Estrous Cycle (the four days of the estrous cycle). Preliminary data analysis revealed no effect of Group (i.e., the four groups of rats that started on different stages of the cycle) on preference scores or intakes and thus it was

not included in the further analysis. The analysis conducted on data from the test with water in both bottles included a factor of Side (left bottle or right bottle). We also examined intakes during the entire day (the sum of all 5 periods) in separate analyses with factors of Cycle and Stage of Estrous Cycle. When main effects or interactions were significant, Tukey's Honestly Significant Difference post hoc tests were used to identify differences between pairs of means. All analyses were conducted using the Statistica software package [20].

Some of the analyses proved uninteresting and so we do not present them here. None of the analyses involving Side or Group produced significant effects: The rats did not have side preferences and the four groups of rats that started on different stages of the cycle had similar intakes. The analyses involving Estrus Cycle Number revealed one complex interaction (rats consumed significantly more bouts of 10 mM CaCl_2 in the first dark period of the 1st cycle than 2nd cycle ($F_{4,64} = 5.4$, $p < 0.001$; 1st cycle = 7.1 ± 0.8 , 2nd cycle = 8.9 ± 0.7). Apart from this, however, calcium intakes during the first and second 4-day cycles were remarkably similar. Consequently, we base the results on average values for the two cycles.

3. Results

3.1. Consumption during the test with two bottles of water

Time of day had a significant influence on water intake: rats consumed more water during the dark than light, and more water during the second half than first half of the dark period (Table S1, Fig.1). Both bout size and bout number were lower during each of the light periods than each of the dark ones (Table S1, Fig.S1–2). Bout size in the middle of the light phase (10:40 to 15:20) was smaller than that during the beginning of the light phase. There were no statistically significant differences in bout size or number between the two dark periods.

Total daily (24-h) water intakes were unaffected by the stage of the estrus cycle (Table 1, Table S2). However, the rats drank significantly less water during the first half of the dark phase of estrus than during the same time of proestrus and diestrus 2 (Table S1, Fig.1). There were no significant differences between the stages of the estrous cycles during the other four time periods. There were also no statistically significant differences in bout size or number related to the stage of the estrous cycle (Table S1, Fig.S1–2).

3.2 Consumption during the test with 10 mM CaCl_2 vs. water

3.2.1. Consumption of 10 mM CaCl_2 —Time of day had a significant influence on calcium solution intake, bout number and bout size (Table S3, Figs.1, S1–2). Rats consumed more CaCl_2 solution during the dark than light. Bout sizes were larger during the two dark periods than during the middle and ending light periods. Rats took more bouts of CaCl_2 solution during each of the dark periods than during each of the light periods and more bouts during the beginning than middle of the light phase.

We did not observe any significant fluctuations in CaCl_2 solution consumption related to the stage of the estrous cycle (Table S3, Fig.1).

Daily (24-h) calcium solution intakes were not affected by estrous cycle (Table 1, Table S4).

3.2.2 Consumption of water when CaCl_2 was available—Water drinking patterns during CaCl_2 solution presentation were similar to those observed during the test with two bottles of water. The rats consumed less water during each of the light periods than during each of the dark ones (Table S3). Water intakes were significantly lower during the middle

(from 10:40 to 15:20) than beginning or end of the light phase; intakes were lower during the beginning than end of the dark phase. Water bout size differed between all five circadian periods such that bout size could be ordered as: Dark 2 > Dark 1 > Light1 > Light3 > Light2. Rats took more water bouts during the dark phase than light phase, more during the end than beginning of the dark phase, and more during the end than middle of the light phase.

Water intake during the first 5 h of the dark phase was lower at estrus than during the same period during other stages (Table S3, Fig.1). The only difference in bout number or size relating to the stage of the estrous cycle was that the number of bouts during the end of the light period of proestrus was significantly lower than that during the end of the light period of diestrus 1 (Fig.S2).

Daily (24 h) water intakes did not change over the estrous cycle (Table 1, Table S4).

3.2.3. Total fluid intake—Fluctuations in total fluid intakes during the test with CaCl₂ solution resembled the pattern of changes in water intakes (Table S3, Fig.1). Total fluid intakes during each of the dark periods were higher than those during each of the light periods. Total intakes at the beginning of the dark phase were lower than intakes at the end. Intakes during the middle of the light phase were lower than those during the beginning and end.

The stage of the estrous cycle influenced total fluid intake only during the first half of the dark period, at which time total fluid intakes were significantly lower during estrus than during any other stage (Table S3, Fig.1).

There were no changes in daily (24-h) total intakes related to the stage of the estrous cycle (Table 1, Table S4).

3.2.4. CaCl₂ preference scores—Preference scores for CaCl₂ solution were unaffected by the time of day or stage of the estrous cycle (Tables S3–4, Fig.1).

4. Discussion

This is the first study to assess calcium intake and preference over the estrous and circadian cycles of the rat. We found that the circadian cycle but not the estrous cycle influenced CaCl₂ intake and neither cycle influenced CaCl₂ preference scores. We did not observe any changes in the CaCl₂ intake microstructure over the estrous cycle.

Over the circadian cycle, fluctuations in calcium solution consumption occurred in parallel with those of water and thus were most likely due to the well-documented circadian fluctuations in fluid intake (e.g., [16,21–23]) rather than a calcium-specific behavior. The lack of any significant changes in preference ratios for calcium solution supports this interpretation.

Over the estrous cycle, water and total fluid intakes fluctuated with the largest perturbation being a reduction during the early dark hours of the estrus stage. This is consistent with several previous findings that estrus exerts robust reductions on consummatory behavior (e.g., [4,24,25]). Despite this decrease in fluid intake during the first dark hours at estrus there were no differences in total 24-h liquid intake among the stages. The decrease in water intake during the first hours of the dark period of estrus parallels data on feeding behavior: during estrus, food intake and meal size are lowest during the first half of the dark period and gradually increase during the second half of the dark period [24,25]. Obviously, CaCl₂ solution is a source of water as well as calcium so one might expect its intake to be governed to some extent by controls of hydration. However, we saw no evidence of this. Either (a)

counteracting factors kept levels of daily calcium solution intake constant despite fluctuations in fluid-drinking behavior, or (b) there was a lack of statistical power related to the small volumes of calcium consumed. The latter possibility is supported to some extent by our observation that there were no changes in CaCl_2 preference scores: indeed if calcium solution intake did not change at estrus when the water intake dropped at least a small increase in preference ratio would be expected. Nevertheless, we lean toward the former possibility because treatment with exogenous estrogen decreases water intake and increases calcium consumption [4,9,10]. Thus, the steady intake of calcium solution could be a result of an interaction of these two effects at estrus.

There is little reason to suspect that our test procedures were insufficiently sensitive to detect changes in need-free calcium intake. Our group size of 17 rats was relatively large for this kind of experiment and our methods detected differences in CaCl_2 solution intake between the middle and the beginning of the light phase. However, there are several limitations of the methodological approach we used: Fluid intakes during the middle of the light period were extremely low and thus if there were any changes in the acceptance of calcium during this period they would be very hard to detect under *ad libitum* conditions (although, for the same reasons, they are also unlikely to be of physiological significance). The rats received adequate amounts of calcium from food so changes in the demand for calcium may have been assuaged from this source. Our results are limited to one concentration of one calcium salt (i.e., 10 mM CaCl_2) and one strain of rat. The last factor is important because there are large differences in calcium acceptance between strains [19,26] and so there could also be strain differences in the patterns of calcium intake during the circadian and estrous cycles.

Although we did not observe fluctuations in calcium intake and selection over the estrous cycle this does not preclude estrogens (or other hormones fluctuating with the estrous cycle) from influencing the desire to consume calcium in other situations (e.g., [4,9,10]). However, most studies addressing the action of reproductive hormones on calcium intake have been aimed at understanding the mechanisms of increased calcium appetite during pregnancy and lactation (e.g. [9]) and so they have involved high dosages of hormones administered daily for extended periods of time. Steroids administered in this manner could accumulate over the course of several days, which contrasts with the transient changes in estrogen levels occurring during the estrous cycle. More importantly the changes in calcium intake during pregnancy are nonspecific and small compared to intake of other compounds and thus are likely to be a result of a general hyperphagia [27].

Our results can be reconciled with previous studies if the tonic effects (as opposed to phasic effects) of reproductive hormones are critical for calcium ingestion. Early work suggested that male and female rats differ in calcium intake, and females decrease calcium intake after neonatal but not adult ovariectomy [28–30]. This points to the potency of organizational effects of reproductive steroids during development rather than to steady effects later in life. We note that, even so, sex differences in calcium intake do not appear to be very robust because they are not always detectable: No sex differences were found in preferences for calcium solutions of 14 rat strains (including the Long-Evans strain used here) [31]. Only 5 out of 40 mouse strains had sex differences in preference scores for calcium solutions [32]. There were no differences between virgin female and male marmoset monkeys in intake of calcium lactate solutions [33]. Thus, if the action of reproductive hormones on calcium intake during the estrous cycle is present it may be masked by other factors, including early steroid experience and current availability of calcium.

As far as we know, there are no reports that describe the use of the TSE Systems Phenomaster apparatus with a focus on fluid intakes (although the same drinking bottles are

used in the TSE Labmaster indirect calorimetry apparatus, which is in widespread use). It is therefore illustrative to compare our results with those collected by other means. We note that our rats had relatively low daily water intakes (i.e., ~18 mL/d) whereas other investigators report intakes of 30 – 50 mL/d. The use of AIN-76A diet and different housing conditions most likely contributed to this difference. Rats and mice fed chows drink almost 50% more water than do cohorts fed semisynthetic diets [34]. Other factors that might have reduced fluid intakes in our experiment include the larger size of the Phenomaster cages, their brighter illumination (due to the cage size and material and open shelving) and the presence of bedding (as opposed to a stainless steel mesh floor) [35]. But arguably the most influential differences are the “spill-proof” Lixit-style valves on the drinking bottles we used. In order to obtain water, a rat must deflect a spindle with its tongue. Perhaps this is sufficiently uncomfortable or requires enough extra effort that it depresses licking behavior. Despite their lower fluid intakes the rats continued to gain weight and cycle, which suggests that they were adequately meeting hydrational requirements. Nevertheless, this observation underscores that drinking is an operant behavior that can be influenced by the effort required to lick.

5. Conclusion

We found that calcium solution intake fluctuated during the circadian cycle in parallel with changes in water intake but preference scores for CaCl_2 solution did not change. We did not detect any changes in calcium solution intake or preference scores related to the stage of the estrous cycle. The intake of CaCl_2 solution remained constant over the estrous cycle despite the sharp decrease in water intake at estrus. Based on these results we conclude that the intake of calcium solution during the circadian cycle is primarily guided by the demands for hydration (because it is a source of water) but there is no indication that the same is true for the estrous cycle. There may be additional factors that help to keep calcium intake close to constant that are either counteracting estrous changes in fluid intake or are not synchronized with the reproductive cycle, or both.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by The National Institute of Deafness and Other Communication Disorders Grant DC-10149. The Phenomaster equipment was provided by a supplement to NIH grant RO1 DK-46791 as part of the American Recovery and Reinvestment Act. We thank Tiffany Aleman and Maya Mudambi for excellent technical assistance.

Dr. Alexander Bachmanov provided useful comments on the earlier version of this manuscript.

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Highlights

- We measured calcium intake and preference scores over the estrous and circadian cycles in the rat.
- CaCl_2 solution intake fluctuated during the circadian cycle in parallel with changes in water intake; the preference scores for CaCl_2 did not change.
- • CaCl_2 solution intake or preference scores did not change over the estrous cycle.

Figure 1.

Mean $\pm$ SE intakes of water and 10 mM CaCl₂, and preference scores for 10 mM CaCl₂ solution at different stages of the estrous cycle. The upper panel describes total intakes from two bottles of water combined. Lowercase letters mark statistically significant differences in water intake between the stages of the cycle within the same time interval. Uppercase letters mark statistically significant differences in total fluid intake between the stages of the cycle. Means sharing the same letter did not differ significantly. There were no statistically significant differences in CaCl₂ solution intake. D1 = diestrus 1, D2 = diestrus 2, P = proestrus, E = estrus.

Table 1

Total 24-h 10 mM CaCl₂ solution and water intakes at different stages of the estrous cycle.

	Diestrus 1	Diestrus 2	Proestrus	Estrus
Consumption during the test with two bottles of water				
Total	18.2 ± 1.4	18.0 ± 1.6	18.7 ± 2.1	18.9 ± 1.5
Consumption during the test with 10 mM CaCl ₂ vs. water ^I				
Water	11.9 ± 1.3	10.7 ± 1.3	10.9 ± 1.2	11.3 ± 1.1
10 mM CaCl ₂	6.0 ± 1.1	6.2 ± 1.4	5.8 ± 1.1	5.1 ± 1.1
Total	17.9 ± 0.9	16.9 ± 1.0	16.8 ± 0.8	16.4 ± 0.8

Values are means ± SEMs, mL.

^I Average of two estrous cycles