

# Anticipatory behavioral rhythm to scheduled glucose availability in rats

## Ritmo comportamental antecipatório à disponibilidade programada de glicose em ratos

Breno Tercio Santos Carneiro<sup>1</sup>, Fabiano Santos Fortes<sup>1</sup>, John Fontenele Araujo<sup>1</sup>

### ABSTRACT

**Background and objective:** Food-anticipatory behavioral rhythms are observed in several species, including mammals. It is reported that acute ingestion of glucose phase-shifts the food-entrainable oscillator (FEO) in rats. Here, we intended to extend the knowledge about the role of glucose on anticipatory behavioral rhythms by submitting rats to a long period of scheduled glucose availability. **Methods:** Adult Wistar rats were under 12:12 hours light-dark cycle for three days with *ad libitum* feeding (Baseline) prior to being submitted to ten days of scheduled glucose restriction (GR) (three hours duration) starting at Zeitgeber time (ZT) 06 and two days of meal (glucose) omission. **Results:** The animals anticipated glucose availability by increasing motor activity in the two hours preceding ZT 06. The anticipatory behavior observed during the glucose restriction persisted on the second day of glucose omission (i.e. when glucose was not offered). That is, this anticipatory behavior was self-sustained, evidencing the true entrainment of the rhythm. **Conclusion:** Glucose ingestion was a sufficient temporal cue for inducing endogenously-generated circadian anticipatory behavior in the rat.

**Keywords:** Glucose/administration & dosage; Circadian rhythm; Feeding behavior; Rats; Models, neurological; Signal transduction

### RESUMO

**Introdução e objetivo:** Ritmos comportamentais antecipatórios ao alimento são observados em várias espécies, incluindo mamíferos. Foi reportado que a ingestão aguda de glicose muda a fase do oscilador sincronizado por ciclo de alimento em ratos. Aqui, pretendeu-se estender o conhecimento sobre o papel da glicose nos ritmos comportamentais antecipatórios submetendo ratos a um longo período de disponibilidade programada de glicose. **Métodos:** Ratos Wistar adultos ficaram sob ciclo claro-escuro de 12:12 horas por três dias com alimentação à vontade (linha de base) antes de serem submetidos a dez dias de restrição de glicose (RG) (três horas de duração), começando na hora do Zeitgeber (HZ) 06 e dois dias de omissão de comida (glicose). **Resultados:** Os animais anteciparam a disponibilidade de glicose aumentando a atividade motora nas duas horas precedentes à

HZ 06. O comportamento antecipatório observado durante a restrição de glicose persistiu no segundo dia de omissão de glicose (i.e., quando a glicose não foi oferecida). Ou seja, este comportamento antecipatório era autossustentado, evidenciando a sincronização real do ritmo. **Conclusão:** A ingestão de glicose foi uma pista temporal suficiente para induzir um comportamento circadiano antecipatório gerado endogenamente no rato.

**Descritores:** Glicose/administração & dosagem; Ritmo circadiano; Comportamento alimentar; Ratos; Modelos neurológicos; Transdução de sinal

### INTRODUCTION

For the organisms living on or near earth's surface, the main *Zeitgeber* is the cyclic alternation between light and dark periods. In mammals, the pacemaker located in the suprachiasmatic nucleus (SCN) is entrained by the light-dark cycle. The output from the SCN to other regions in the brain leads to the expression of behavioral and physiological parameters adjusted to long day (LD) <sup>(1)</sup>. It has been reported, however, that under conditions of scheduled restricted feeding, clock gene oscillations in different brain regions and in peripheral tissues become entrained to feeding time <sup>(2-6)</sup>.

During periods of scheduled restricted feeding, rats and other species show increased activity (e.g. locomotion, wheel-running activity, food-bin activity) in the two to three hours preceding food availability <sup>(7,8)</sup>. Under natural conditions, periods of food scarcity may occur. In this situation, it must be important for species to be behaviorally and physiologically prepared when food sources become available in a determined time of day.

The anticipation to feeding time was named food-anticipatory activity (FAA) in early years, and its oscillatory and circadian properties were well demonstrated <sup>(7,8)</sup>. The loca-

Study carried out at Laboratório de Neurobiologia e Ritmicidade Biológica, Departamento de Fisiologia, Universidade Federal do Rio Grande do Norte – UFRN, Natal (RN), Brazil.

<sup>1</sup>Laboratório de Neurobiologia e Ritmicidade Biológica, Departamento de Fisiologia, Universidade Federal do Rio Grande do Norte – UFRN, Natal (RN), Brazil.

**Corresponding author:** Breno Tercio Santos Carneiro – Centro de Biociências, Departamento de Fisiologia, Universidade Federal do Rio Grande do Norte – Campus Universitário, Lagoa Nova – Caixa Postal 1506 – CEP 59078-970 – Natal (RN), Brazil – Phone: (84) 3215-3409 (Ext 218); Fax: (84) 3211-9206 – E-mail: brenotercio@yahoo.com.br, brenotercio@cb.ufrn.br.

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tion of the oscillator responsible for this circadian behavior has been investigated during the last 30 years. In late 1970's, Stephan et al.<sup>(9)</sup> first showed that the pacemaker entrained by light located in the SCN was not necessary for the expression of food-anticipatory activity. Since then, many investigators have tried to locate the so-called food-entrainable oscillator (FEO) in central or peripheral tissues (3,7,8,10). Nonetheless, the search is still intense and discussions on the organization of the FEO have increased in the last years<sup>(10-12)</sup>. Besides the importance of the location of the FEO, another important question to be answered is: what are the mechanisms by which the anticipatory behavioral rhythm is entrained? In other words, what are the signals associated with feeding that transmit information to the brain about scheduled food availability?

In face to the relevance of different humoral signals acting in many brain sites to regulate food intake, their daily fluctuation and, more importantly, their fluctuation in response to feeding schedules, we recently proposed that food-anticipatory behaviors would be generated by the coordinated activity of different brain areas which are directly or indirectly entrained by an array of humoral signals which have their concentration cycling in response to food ingestion. These include both orexinergic and anorexigenic signals in the blood<sup>(11)</sup>.

It has been reported that a minimum caloric content, but not gastric stuffing, is a necessary cue for inducing food-anticipatory behavior<sup>(13,14)</sup>. Stephan and Davidson<sup>(15)</sup> also showed that, despite high caloric content of mineral and vegetable oil, they are not capable of phase-shifting the food-entrainable oscillator as glucose is. These authors initially entrained rats to scheduled regular chow and then phase-delayed the feeding time in eight hours. On the first two days of delay, rats received mineral or vegetable oil, saccharin or glucose+saccharin. The animals receiving glucose showed larger phase-delays and reentrained faster to meal-time, which suggests a role for this nutrient in the oscillatory mechanism of the FEO. Based on our proposal, in this study we intended to test one of the signals that might be involved in timing the FEO in the brain (i.e., glucose) by submitting rats to scheduled glucose availability in the middle of light phase for a long period (ten days).

## METHODS

The research was approved by the Ethics Committee for Use of Animals of Universidade Federal do Rio Grande do Norte (protocol n° 026/2009). Ten 5-month-old Wistar rats (five males and five females), at the beginning of the experiment, were used. The animals were single-housed in polypropylene cages (32x40x17 cm) inside ventilated wooden boxes with controlled temperature ( $23\pm 1^\circ\text{C}$ ) and light-dark cycle

(12:12 hours). Recordings of the motor activity started at ZT 11.5 (by convention, ZT 12 is defined as the time of lights-off) on day 0 (Figure 1).

Under a 12:12 hours light-dark cycle (lights-on at 6h), the animals were submitted to three days of Baseline with *ad libitum* tap water and rodent chow (Labina, Purina®). The chow was withheld at ZT 11.5 on day 3. From day 4 to 13, without access to regular chow, each rat had 20 mL of glucose solution (50%) (Isofarma®, Industrial Farmacêutica Ltda.) in two plastic flasks of 10 mL available from ZT 06 to ZT 09 (Glucose restriction). Then, the glucose solution was not offered for two days (14 and 15) (Omission). To allow the animals to learn how to drink in the glucose plastic flasks, 10 mL was offered at ZT 11.5 and remained available for 24 hours on days 0, 1 and 2.

The motor activity rhythm was recorded by infrared motion sensors positioned 15 cm above the cage lids connected to a personal computer where data were collected each 5 minutes using *Aschoff* software. Motor activity data were transformed in percentage of total daily activity. Anticipation was defined as the amount of activity during the two hours preceding the glucose availability (i.e. between ZT 04 and ZT 06). Actograms were drawn on *El Temps* software (A. Díez-Noguera, Universitat de Barcelona, 1999). For statistical analyses, repeated measures ANOVA followed by Tukey HSD test, *t* test for dependent samples and Pearson's correlation were performed on *Statistica 7* software. Analyses were considered statistically significant when  $p < 0.05$ . Data are expressed as means  $\pm$  standard error of mean (SEM).

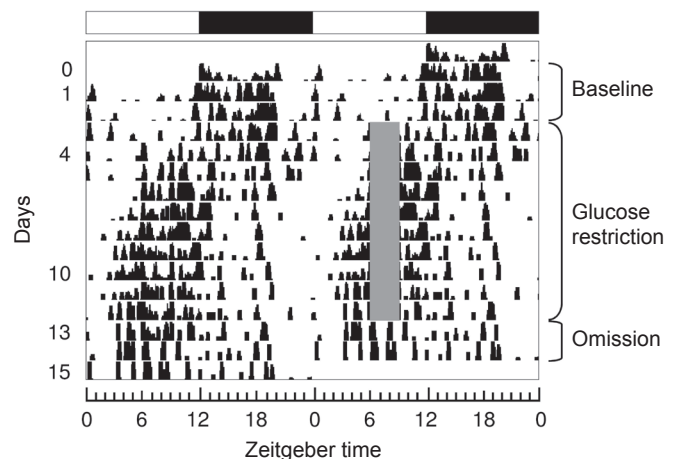
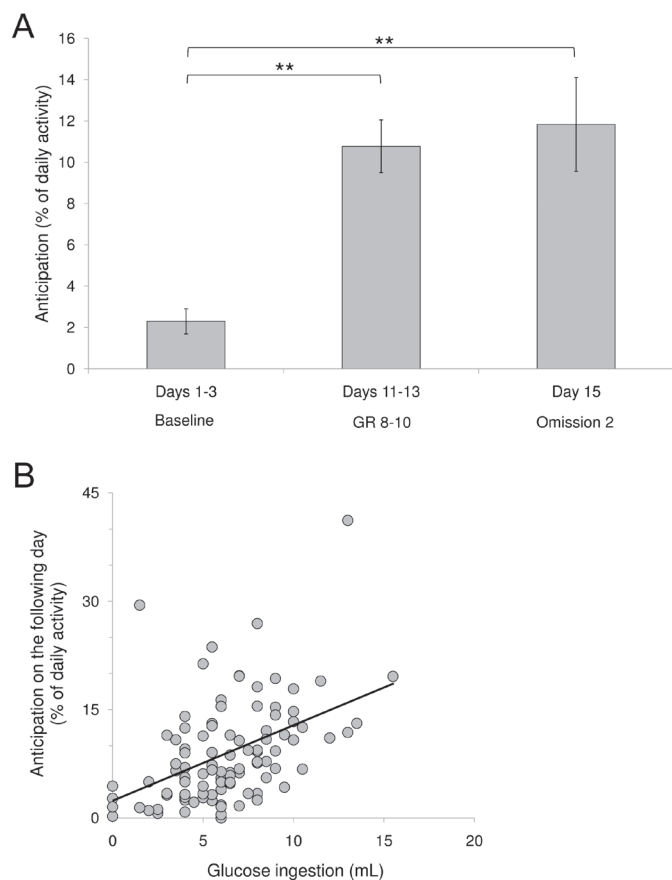


Figure 1: A double-plotted actogram of the motor activity rhythm from a representative rat. *Zeitgeber time* (ZT) is indicated at the bottom. Bar indicating the light-dark cycle is shown on the top. Days 1 to 3 were Baseline. Glucose restriction (vertical grey bar, ZT 06-09) started on day 4 and finished on day 13. On days 14 and 15, the glucose solution was not offered (omission). Anticipatory behavior is seen during glucose restriction and on days of omission.

## RESULTS

The animals learned how to drink the glucose solution in their flasks. Glucose ingestion was  $2.85 \pm 0.67$  mL on day 4 and  $7.7 \pm 0.73$  on day 13 ( $p < 0.001$ ,  $t$  test for dependent samples). An anticipatory behavioral rhythm is evidenced by



\*\* $p < 0.01$ , *post-hoc* Tukey HSD test after ANOVA for repeated measures.

**Figure 2:** (A) Averaged motor activity between ZT 04-06 (anticipation) over three days of baseline and glucose restriction (GR) and on omission 2nd day. High level of motor activity is evident in the last three days of GR and this anticipatory behavior persists on the second day of meal omission. (B) Correlation between glucose ingested and the anticipation on the next day.  $R = 0.445$ ,  $p < 0.001$ , Pearson's correlation.

visual inspection of the actogram (in this representation, the days are xy graphs of 48 hours plotted sequentially from the top to the bottom) on Figure 1.

Figure 2A shows averaged anticipation over the baseline days and the last three days of glucose restriction and on the second day of omission. Gathering data from all days of glucose restriction, we found a statistically significant correlation between glucose ingestion and the level of anticipation on the next day ( $R = 0.445$ ,  $p < 0.001$ , Pearson's correlation) (Figure 2B).

## DISCUSSION

We found that scheduled glucose ingestion for ten days is a cue sufficient to induce anticipatory behavior in rats. Also, the volume ingested was correlated with the anticipatory behavior observed, corroborating the hypothesis that the caloric content is necessary for the expression of food-anticipatory behaviors<sup>(13)</sup>.

The most common measure for observing food anticipation is the locomotor behavior in a running wheel. In rats with scheduled food availability in the middle of the light phase, for example, a pronounced increase in wheel-running activity is observed at the hours preceding feeding time, almost no running after, and again high activity levels in the dark phase, mainly in the first half. The pattern observed in general motor activity is a bit different from that in a running wheel. As the motion sensor detects all kinds of movement (locomotion, feeding, grooming), the records are more spread. Most of the animals in this study showed a pattern similar to Figure 1, with high levels of activity after the glucose availability. Some showed a pattern more similar to that observed in running wheels, with a lower level of activity when compared to other animals in the hours succeeding glucose availability. Regarding the anticipatory behavior, we observed that it is not different between rats anticipating glucose and rats anticipating chow at the same circadian phase (unpublished data).

Mistlberger et al.<sup>(16)</sup> reported that feeding paradigms of protein and fat or protein and carbohydrate, each one available for one hour daily in the light phase and separated by seven hours, induces anticipatory food-bin activity in rats. They also showed that these macronutrients were ineffective to induce anticipation if the animals were not under caloric restriction. Later, Stephan and Davidson<sup>(15)</sup> showed that glucose ingestion, but not other caloric substances (mineral or vegetable oil), rapidly phase-shifts the food-entrainable oscillator in rats. This suggests that glucose concentration could directly alter the activity of the food-entrainable oscillator. Alternatively, as a consequence of glucose ingestion, the concentration of other humoral signals (e.g. hormones) would change and this could serve as a phase-shifting cue for the FEO, or the two processes could occur simultaneously. The observation by Stephan and Davidson that fat ingestion is not effective in phase-shifting the FEO might seem contradictory to the Mistlberger et al. report that fat induces anticipatory behavior. However, we should consider methodological differences in the two studies. Rats in Mistlberger et al. study were maintained on the feeding paradigm for 14 days. In Stephan and Davidson study, however, the animals were fed for only two days with fat and phase-shifts were measured. In the second study, the rats were maintained on the feeding paradigm for 14 days. It is possible that fat ingestion triggers the signals necessary to entrain the FEO in a less

effective manner, thus being required a longer period to observe anticipation, and consequently phase-shifts would not be evident in only two days of ingestion. On the other hand, glucose ingestion could be more effective in altering the internal signals necessary for entrainment (the rise in plasma glucose itself, for example). It is not completely described, actually, which signals associated with food ingestion are necessary or indispensable for entrainment of anticipatory circadian behaviors.

In a recent report, Waddington Lamont et al. <sup>(2)</sup> showed that rats receiving sucrose (fructose+glucose) in the middle of the light phase do not express anticipatory behavior. Again, methodological differences must be accounted. Here, we used concentrated glucose at 50% while they used sucrose at 32%; in the present study, the behavioral measure was the general motor activity while Waddington Lamont et al. used wheel-running activity; and the most important difference we consider is that in the study by Waddington Lamont et al., the animals had chow *ad libitum* plus sucrose at midday while our rats had glucose as their only food source. We discussed that a caloric restriction is required for the expression of food-anticipatory behaviors <sup>(16)</sup>, thus the absence of anticipatory behavior in Waddington Lamont et al. study could be due to the absence of caloric restriction, since their rats had chow *ad libitum*. The same research group has, actually, reported later that a highly palatable food does not induce anticipation unless the animal is in a negative metabolic state <sup>(17)</sup>.

Blood glucose is one of the humoral parameters that change in response to food ingestion. It rises specifically after a meal. In this paper, we could not attribute the entrainment of anticipatory behavior to glucose only, since during the glucose restriction period other signals might contribute in some way for its expression. Some of these signals have been shown to fluctuate in response to scheduled feeding (e.g., ghrelin, leptin, glucagon, insulin) <sup>(18-20)</sup> and it is likely that they would also respond to long-term scheduled glucose ingestion. Also, it has been shown that some of these signals affect variables that entrain to mealtime and/or modify the expression of food-anticipatory behaviors <sup>(4,21,22)</sup>. Our hypothesis is that the change in the concentration of a range of humoral signals (related to the regulation of food intake) after a meal is responsible for timing the food-entrainable oscillator in the brain, which is responsible for the expression of anticipatory behavioral rhythms <sup>(11)</sup>.

Regarding glucose itself, it has been shown that its extracellular concentration alter the firing rate of neurons in important hypothalamic regions involved in the regulation of food intake, promotion of arousal and which entrain to feeding schedules, such as the arcuate nucleus (ARC), the ventromedial nucleus (VMN) and the lateral area <sup>(23)</sup>. This

points to the relevance that the fluctuation in glucose concentration probably has in timing different brain regions. A recent work has suggested a role for the VMN in the initiation of the food-anticipatory behavior <sup>(24)</sup>. Previous reports have also pointed the importance of the LH on the expression of food anticipation <sup>(25)</sup>. We must mention, however, that there are controversies regarding the importance of the integrity of these and many other areas for the expression of food-anticipatory behavioral rhythms <sup>(11)</sup>. The results obtained in most lesion studies accomplished until now should indicate that the FEO, instead of constituted by one single area in the brain, is rather composed of a network of different areas <sup>(11)</sup> or an emergent oscillator born due to the feeding/fasting cycle present during feeding schedules <sup>(12)</sup>.

Reports discussed above point that highly palatable food associated with food deprivation induces anticipatory behaviors in rats. In this study, we eliminated any other nutrient from feeding and showed that glucose is able to induce similar food-anticipatory rhythms. We could say that a rise in plasma glucose constitutes at least one of the signals responsible for entrainment of anticipatory behavioral rhythms. Perhaps, the change in the concentration of many humoral signals due to food intake is the entraining cue to the food-entrainable oscillator in the brain <sup>(11)</sup>.

We were able to show that glucose ingestion is a temporal cue sufficient to induce anticipatory behavioral rhythm in rats without access to any other food source and that anticipation varies as a function of volume ingested and, consequently, the caloric content.

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