Published in final edited form as:

Eur J Neurosci. 2012 May ; 35(9): 1458–1465. doi:10.1111/j.1460-9568.2012.08051.x.

Synchronization of PER1 protein in Parabrachial nucleus in a natural model of food anticipatory activity

Claudia Juárez¹, Elvira Morgado¹, Stefan M. Waliszewski², Armando J. Martínez³, Enrique Meza¹, and Mario Caba¹

¹Centro de Investigaciones Biomédicas, Universidad Veracruzana, A.P. # 114, Xalapa, Ver., México

²Instituto de Medicina Forense, Veracruz, Ver

³Instituto de Neuroetología, Universidad Veracruzana, Xalapa, Ver., México

Abstract

Rabbit pups represent a natural model of food anticipatory activity (FAA). FAA is the behavioral output of a putative food entrainable oscillator (FEO). It had been suggested that the FEO is comprised of a distributed system of clocks that work in concert in response to gastrointestinal input by food. Scheduled food intake synchronizes several nuclei in the brain, and the hypothalamus has received particular attention. On the contrary, brainstem nuclei, despite being among the brain structures to first receive food cues, have been scarcely studied. Here we analyzed by immunohistochemistry possible oscillation of FOS and PER1 proteins through a complete 24 h cycle in the dorsal vagal complex (DVC) and parabrachial nucleus (PBN) of seven to eight day old rabbit pups scheduled to nurse during the night (02:00) or day (10:00) and also in fasted subjects to explore the possible persistence of oscillations. We found a clear induction of FOS that peaks 1.5 h after nursing in all nuclei studied. PER1 was only synchronized in the PBN, reaching highest values 12 h after nursing. Only PER1 oscillations persisted in fasted subjects. We conclude that the DVC nuclei are probably more related to the transmission of food cues to other brain regions but that the PBN participates in the integration of information essential for FAA. Our results support previous findings suggesting that the DVC nuclei, but not PBN, are not essential for FAA. We suggest that PBN is a key component of the proposed distributed system of clocks involved in FAA.

Keywords

synchronization by food; anticipatory arousal; clock genes; FOS protein; nucleus tractus solitarius

Introduction

Rabbit pups are a natural model of food restriction since they ingest food, in this case, milk, just once a day and subjects show behavioral food anticipatory activity (FAA) to this event (Jilge, 1993; Mistlberger 1994; Caba et al., 2008; Morgado et al., 2011). Though this daily nursing episode is brief, lasting less than 5 min., pups ingest up to 35% of their body weight in milk at postnatal day seven (Caba et al., 2003). This cyclic food ingestion imposes a rhythm in metabolites as glucose, liver glycogen and free fatty acids and also in the hormones ghrelin and corticosterone, which all shift their rhythm in parallel to timing of milk ingestion (Morgado et al., 2008; 2010). Similar to rabbit pups, rats under a food

*Correspondence: Dr. Mario Caba, as above. mcaba@uv.mx.

restriction schedule also develop FAA before food presentation, which is considered the behavioral output of a putative food entrainable oscillator (FEO; Stephan, 2002). Periodic food ingestion plays an important role in food entrainment as part of the synchronizing process necessary to establish FAA. In this regard the digestive system develops food-driven rhythms (Davidson et al., 2003) and cerebral structures in the hindbrain receives food cues, which are transmitted to several regions in the brain, particularly hypothalamic nuclei where the FEO had been the subject of intense search (Davidson, 2009; Landry et al., 2011). However, the locus of the FEO, if any, had not been found and in the hypothalamus several nuclei show activation before, during and after food presentation (Angeles-Castellanos et al., 2004). Moreover, it had been suggested that rather a single locus, FAA probably depends on a distributed system of clocks that work in concert (Davidson, 2009). In contrast to the hypothalamus, few studies have focused on brain stem nuclei, which as mentioned, receive periodic food cues and play a relevant role in the input mechanism of the FEO. In this regard, in the rat, electrolytic and neurochemical lesions of the parabrachial nucleus (PBN) in the hindbrain markedly attenuated and in some cases abolished FAA (Davidson et al., 2000). PBN receives dense afferents from nucleus of tractus solitarius (NTS) and area postrema (AP), and it sends projections to hypothalamic nuclei involved in the control of food intake (Berthoud, 2002). Additionally NTS send projections to motor dorsal nucleus of the vagus (DMX), which through parasympathetic preganglionic afferents modulates the digestive system (Rogers & Hermann, 1992). NTS, AP and DMX form the dorsal vagal complex (DVC) which together with the PBN plays a key role in the central control of ingestive behavior by modulating neural signaling from the gastrointestinal system and forebrain (Rinaman, 2006).

By using protein FOS, product of the *c-fos* gene, it had been demonstrated that feeding induces this protein in DVC in the hindbrain (Fraser et al., 1995; Willing & Berthoud, 1997; Rinaman et al., 1998;). Moreover, in the rat model to induce FAA, a 2h food pulse induces transiently high levels of FOS in lateral portion of PBN (Angeles-Castellanos et al., 2005), which reinforces the proposal that this nucleus is important for FAA (Davidson et al., 2000). But to date there is no information about clock genes and their protein products in the PBN or DVC in the food entrainment model to induce FAA.

Rabbit pups offer an extraordinary opportunity to explore the effect of food ingestion on hindbrain nuclei and their role in synchronization, which leads to FAA, for the following reasons. Rabbits ingest food, in this case milk, just once a day (Jilge, 1993) and in less than five minutes ingest up to 35% of their body weight in milk (Caba et al., 2003) as already mentioned. For this reason in the rabbit it is not necessary to deprive pups from suckling milk as in the neonatal rat (Hironaka et al., 2000; Morales et al., 2008) or food in adult rodents (Emond et al., 2001; Angeles-Castellanos et al., 2005) because in the rabbit this is a once a day event that occurs with circadian periodicity (Jilge, 1993). Finally recently it was demonstrated that indeed food, and no other maternal cue during nursing, is a sufficient entraining signal to induce FAA in neonatal rabbit pups (Morgado et al., 2011). Together this evidence supports the proposal of the rabbit pup as a natural model of food entrainment.

In present study we explore the effect of daily milk ingestion on DVC and PBN throughout an entire 24 h cycle before and after milk intake by analyzing induction of FOS protein as an index of neural activation (Morgan & Curran, 1991) and protein PER1, product of clock gene *Per1* as a reporter of oscillatory mechanisms (Angeles-Castellanos et al., 2007; Caba et al., 2008). Furthermore in order to explore possible persistence of oscillations we additionally analyzed both proteins in another entire 24 h cycle in fasted subjects.

Materials and methods

Animal housing

New Zealand white female rabbits, bred in our colony in Xalapa, México, were maintained under a controlled light/dark cycle (12/12 h; lights on at 07:00 h), fed with rabbit pellets (Purina) and water *ad libitum*. Females were housed individually in steel cages at room temperature of $23 \pm 1^\circ\text{C}$ and were monitored daily from day 28 of pregnancy until delivery. The cage consisted of three compartments, one for the mother, one for the nest and a tunnel between them; this latter had a sliding door in order to control the access of the mother to the nest (Caba et al., 2008). After birth litters were adjusted to 7–8 pups and remained in the nest compartment, in which pups were kept in constant darkness and undisturbed for the entire experiment. To determine that pups were synchronized to daily food intake, their locomotor behavior was monitored in the nest with a detector sensitive to pup's movements through the infrared radiations emitted by their body (Morgado et al., 2010). All experimental procedures were approved by the Ethics committee of Universidad Veracruzana, in accordance with the procedures of the National Guide for the Production, Care and Use of Laboratory animals (Norma Oficial Mexicana NOM-062-ZOO-1999), which complies with international guidelines of the Society for Neuroscience on the ethical use of animals.

Experimental design

On the day of parturition (postpartum day 0; PD0) the door between the mother's compartment and tunnel was locked and starting the next day (PD1) was opened either at 02:00 or 10:00 h from PD1 to PD7. At PD7 pups were assigned to one of both groups, Restricted Food (RF) 02:00 or RF10:00 on basis of their time of nursing. Nursing time was considered zeitgeber (ZT) 0 and pups were sacrificed at ZT0, 1.5 h after nursing (ZT1.5) and then at 4-h intervals at ZT4, ZT8, ZT12, ZT16 and ZT20 ($n=4$ at each time point). To explore persistence of possible oscillations additional subjects were fasted (restricted feeding-fasted; RF-F) at PD8 for one nursing bout and sacrificed starting after expected nursing and then at similar intervals as nursed subjects ($n=4$ at each time point), either in pups nursed at 02:00 (RF-F02:00) or 10:00 h (RF-F10:00).

Immunohistochemistry

Rabbit pups were anesthetized with an overdose of sodium pentobarbital (20 mg per pup i.p.) and were perfused transcardially with saline solution (0.9%), followed by 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). The brains were removed immediately after perfusion, cryoprotected successively in 10, 20 and 30% sucrose in PB and sectioned coronally at $50\ \mu\text{m}$ in a cryostat (Microm). Serial sections were collected in PB at the level of the hindbrain. Every two of four sections were used for labeling of FOS and PER1, following previous protocols for FOS (Caba et al., 2003) and PER1 (Caba et al., 2008) in rabbit pup brain. Tissue was washed in PB of 5 min each and then exposed for 10 min in 0.5% hydrogen peroxide solution to eliminate endogenous peroxidase activity. Free floating sections were incubated in FOS antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or PER1 antibody (sc-7724, Santa Cruz Biotechnology, Santa Cruz, CA, USA) both diluted at 1:2000, in 3% normal horse serum and 0.3% Triton X-100 (Sigma), for 48 h at 4°C . Tissue was incubated in biotinylated secondary antibody, horse anti-goat diluted at 1:200 (Vector Laboratories) in PB and 0.3% Triton X-100 for 1 h at room temperature, and then in avidin-biotin-HRP complex diluted at 1:200 (Vector Laboratories) in PB at room temperature. Between incubations, tissue was washed four times for 10 min in PB. Both, FOS and PER1 antibody-peroxidase complex was stained with a solution of 0.05% diaminobenzidine (Polysciences) in the presence of nickel sulfate (10 mg/ml, Fisher Scientific), cobalt chloride (10 mg/ml, Fisher Scientific) and 0.01% hydrogen peroxide to

obtain a black-purple precipitate. After 10 min, tissue was transferred to PB in order to stop the reaction. Sections were mounted onto gelatin-subbed slides, dehydrated and coverslipped with Permount. In all cases, tissue sections from subjects of each of the different time points were processed together. Control sections were processed as above but with the primary antibody omitted. Additionally, sc-7724 PER1 and sc-52 FOS antibodies had been characterized and tested in rabbit tissue (Caba et al., 2008).

Quantification of immunostaining

FOS- and PER1-immunoreactivity (-ir) were identified by the presence of a black-purple pitate from the DAB-nickel/cobalt reaction in the cell nucleus. Positive cells were determined by comparing labeling with the background optical density in a nearby region lacking immunoreactivity with the Image Pro Plus v. 5 (Media Cybernetics, Silver Spring, MD, USA) attached to a light microscope (Olympus BX41). Immunoreactive cells that reached five times the optical density background level were considered positive, and those below this staining level were considered negative. Numbers of FOS- and PER1- ir cells were quantified using a grid by two observers blind to the experimental condition of the subjects according to a protocol described previously (Caba et al., 2008). NTS, DMX and AP were analyzed at level No. 4 and PBN at level No. 19 of a topographic description of the rabbit brainstem (Pau et al., 1997), which are similar to plates IV and XIII, respectively of a cytoarchitectonic atlas of the rhombencephalon of the rabbit (Messen & Olzewski, 1949).

Statistical analysis

A two-way ANOVA for the main effects of group condition, time factor and the interaction between group and time was carried out, in which the dependent variables were the state of feeding, either nursing or fasting, while the independent variable was the time at which pups were nursed or fasted. This analysis was followed by a post-hoc Tukey-Kramer test for 2×2 comparison of equivalent time in both conditions. In order to not violate the assumptions of homogeneity of variance, in all cases, data were rank-transformed before ANOVA (Conover & Iman, 1981; Morgado et al., 2010; 2011). Statistical analyses were performed using Sigma Stat Statistical Software version 3.5. Probability levels of $P < 0.05$ were considered significant. Values given are means $\pm$ SEM. and they were plotted without transformation.

Results

Locomotor behavior

Pups developed FAA as revealed by an increase in locomotor behavior before scheduled nursing in both pups nursed during the night (RF-02:00) and the day (RF-10:00). In Fig. 1 we present a waveform of all litters. Note that locomotor behavior increased around 3 h before nursing, was maximal at nursing time, then decreased and remained low in the time interval between nursing bouts.

Mean body weight of groups at the time of sacrifice was: RF02:00 = 96.91 ± 15.7 g; RF10:00 = 101.94 ± 15.9 g ($P < 0.05$, RF02:00 vs RF10:00); RF-F02:00 = 79.11 ± 8.76 , RF-F10:00 = 82.5 ± 5.41 g ($P < 0.05$, RF-F02:00 vs RF-F10:00).

FOS-ir in NTS, DMX, AP and PBN

In both night and day nursed subjects FOS expression followed a rhythm in all structures, which shifted in parallel to timing of nursing. After suckling milk there was a sharp increase, which declined to reach basal levels around 8 h after suckling and remained low until next nursing. On the contrary, this rhythm did not persist in fasted subjects where FOS levels were very low in most time point groups. In Fig. 2A we show a representative photomicrograph of FOS expression in NTS.

FOS-ir in NTS

Subjects nursed at 02:00 h—Quantitative analysis indicated that in nursed and fasted pups, FOS expression in the NTS varied significantly with group condition ($F_{1,55} = 101.56$, $P < 0.001$), time factor ($F_{6,55} = 17.89$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 48.47$, $P < 0.001$) (Fig. 3A). In the RF02:00 group, the highest FOS expression at ZT1.5 was significantly different than values at ZT08, ZT12, ZT20 ($P < 0.001$ in all cases), ZT0 ($P = 0.002$) and ZT16 ($P = 0.038$). In the RF-F02:00 group, the highest expression of FOS was observed at ZT08 ($P < 0.001$ against all values).

Subjects nursed at 10:00 h—Quantitative analysis indicated that in nursed and fasted pups, FOS expression in the NTS varied significantly with group condition ($F_{1,55} = 21.64$, $P < 0.001$), time factor ($F_{6,55} = 52.02$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 17.09$, $P < 0.001$) (Fig. 3B). In RF10:00 group, the highest value at ZT1.5 was significantly different than values at ZT08, ZT20 ($P < 0.001$ in both cases) and ZT12 ($P = 0.007$). Additionally, values at ZT0, ZT04 and ZT20 were different of those at ZT08, ZT12 and ZT16 ($P < 0.001$ in all cases). In RF-F10:00, the highest expression of FOS at ZT0 was significantly different than remaining time point values ($P < 0.001$ in all cases).

FOS-ir in DMX

Subjects nursed at 02:00 h—Quantitative analysis indicated that in nursed and fasted pups, FOS expression in the DMX varied significantly with group condition ($F_{1,55} = 12.75$, $P < 0.001$), time factor ($F_{6,55} = 14.39$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 32.53$, $P < 0.001$) (Fig. 3C). In the RF02:00 group, the value at ZT0 was significantly higher than ZT12, ZT16 and ZT20 ($P < 0.001$ in all cases); in addition, the value at ZT08 was higher than that at ZT12 and ZT16 ($P < 0.001$ in both cases). In RF-F02:00 group, the highest expression of FOS at ZT16 was significantly different than values at ZT0, ZT1.5, ZT04, ZT12 ($P < 0.001$ in all cases) and ZT20 ($P = 0.004$).

Subjects nursed at 10:00 h—Quantitative analysis indicated that in nursed and fasted pups, FOS expression in the DMX varied significantly with group condition ($F_{1,55} = 14.17$, $P < 0.001$), time factor ($F_{6,55} = 24.03$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 16.48$, $P < 0.001$) (Fig. 3D). In the RF10:00 group, the highest values at ZT1.5 and ZT04 were significantly different than those values at ZT08, ZT12, ZT16 ($P < 0.001$ in all cases). In the RF-F10:00 group, the highest FOS expression at ZT0 was significantly different than values at ZT1.5, ZT08, ZT12 ($P < 0.001$ in all cases), ZT04 ($P = 0.001$), ZT16 ($P = 0.004$) and ZT20 ($P = 0.008$).

FOS-ir in AP

Subjects nursed at 02:00 h—Quantitative analysis indicates that in nursed and fasted pups, there was no significant effect of group condition ($F_{1,55} = 0.04$, $P = 0.834$), but there was a significant difference in the time factor ($F_{6,55} = 5.91$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 47.03$, $P < 0.001$) (Fig. 3E). In the RF02:00 group, the highest FOS expression at ZT1.5 was significantly different than values at ZT08, ZT12, ZT16, ZT20 ($P < 0.001$ in all cases) and ZT0 ($P = 0.022$). In the RF-F02:00 group, the highest expression of FOS at ZT20 and ZT08 were significantly different than values at ZT1.5 ($P < 0.001$) ZT04 and ZT0 ($P = 0.001$ in both cases).

Subjects nursed at 10:00 h—Quantitative analysis indicated that in nursed and fasted pups, there was no significant effect of group condition ($F_{1,55} = 2.45$, $P = 0.125$), but there was a significant difference in the time factor ($F_{6,55} = 13.34$, $P < 0.001$) and the interaction

between feeding condition and time ($F_{6,55} = 15.42$, $P < 0.001$) (Fig. 3F). In the RF10:00 group, the highest values of FOS expression at ZT1.5, ZT04 and ZT0 were significantly different than values at ZT12, ZT16, ZT20 ($P < 0.001$ in all cases) and ZT08 ($P < 0.001$; $P = 0.007$; $P = 0.043$, respectively). In the RF-F10:00 group, the highest FOS expression at ZT12 was significantly different than values at ZT0 ($P = 0.004$), ZT08 and ZT20 ($P = 0.016$, in both cases).

FOS-ir in PBN

Subjects nursed at 02:00 h—Quantitative analysis indicated that in nursed and fasted pups, FOS expression in the PBN varied significantly with group condition ($F_{1,55} = 61.06$, $P < 0.001$), time factor ($F_{6,55} = 60.20$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 18.31$, $P < 0.001$) (Fig. 3G). In the RF02:00 group, the highest values at ZT1.5 and ZT04 were significantly different compared to remaining time point values ($P < 0.001$ in all cases). In the RF-F02:00 group, values at ZT1.5 and ZT04 were significantly different than those at ZT0 ($P < 0.001$ in both cases) ZT16 ($P < 0.001$; $P = 0.007$, respectively) and ZT08 ($P = 0.001$; $P = 0.040$, respectively).

Subjects nursed at 10:00 h—Quantitative analysis indicated that in nursed and fasted pups, FOS expression in the PBN varied significantly with group condition ($F_{1,55} = 54.11$, $P < 0.001$), time factor ($F_{6,55} = 30.57$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 23.75$, $P < 0.001$) (Fig. 3H). In the RF10:00, the highest expression of FOS at ZT1.5 and ZT04 was significantly different than remaining time point values ($P < 0.001$ in all cases). In RF-F10:00 group, values at ZT1.5, ZT12 and ZT16 were significantly different than the value at ZT0 ($P = 0.006$; $P = 0.040$; $P = 0.047$).

PER1-ir in NTS, DMX, AP and PBN

In NTS, DMX and AP, in both night and day nursed subjects, PER1 protein was expressed without an apparent rhythm. Concurrent timing of nursing seems does not affect the induction of this protein as revealed by their different levels in night and day fed groups at the same time point. In fasted subjects we found different levels of expression of PER1 also without an apparent rhythm between nursing conditions and timing of sampling (data not shown). On the contrary, in the PBN we found a clear pattern of PER1 induction that shifted in parallel to the timing of nursing. In Fig. 2B we show a representative photomicrograph of PER1 expression in PBN.

PER1-ir in PBN

Subjects nursed at 02:00 h—Quantitative analysis indicated that in nursed and fasted pups, PER1 expression in the PBN varied significantly with group condition ($F_{1,55} = 10.80$, $P = 0.002$), time factor ($F_{6,55} = 15.65$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 58.94$, $P < 0.001$) (Fig. 4A). In the RF02:00 group, the highest PER1 expression at ZT12 was significantly different than remaining time point values ($P < 0.001$ in all cases). In the RF-F02:00 group, the highest PER1 expression at ZT0 and ZT1.5 were significantly different than values at ZT12, ZT16 ($P < 0.001$ in both cases), ZT08 ($P = 0.004$), ZT20 ($P = 0.026$) and ZT04 ($P = 0.030$).

Subjects nursed at 10:00 h—Quantitative analysis indicated that in nursed and fasted pups, PER1 expression in the PBN varied significantly with group condition ($F_{1,55} = 6.49$, $P = 0.015$), time factor ($F_{6,55} = 21.78$, $P < 0.001$) and the interaction between feeding condition and time ($F_{6,55} = 36.34$, $P < 0.001$) (Fig. 4B). In the RF10:00 group, the highest PER1 expression at ZT12 was significantly different than remaining time point values ($P <$

0.001 in all cases). In the RF-F10:00 group, the value at ZT1.5 was significantly higher than values at ZT0, ZT08 ($P < 0.001$ in both cases), ZT16 ($P = 0.002$) and ZT20 ($P = 0.004$).

Discussion

We found that ingested milk is a strong signal that produces a rapid induction of FOS protein in all brain stem nuclei analyzed, and which does not persist in fasted condition. Besides that, there is an induction of PER1 that peaks 12 h after milk ingestion only in PBN. However, interestingly, in fasted subjects, PER1 induction reaches a peak at 1.5 h after expected time of scheduled nursing. Our results suggest that brain stem nuclei transmit gastrointestinal information from periphery to forebrain, and, in addition, that the PBN seems to play an important role in the integration of gastrointestinal information necessary for FAA.

Previous studies indicate that circadian milk ingestion is a strong synchronizer of behavioral, hormonal and metabolic parameters in rabbit pups (Rovirosa et al., 2005; Caba et al., 2008; Morgado et al., 2008; 2010). In the brain this periodic signal activates specific neural structures, as the supraoptic (SON), paraventricular (PVN) and dorsomedial (DMN) nuclei in the hypothalamus as revealed by induction of FOS and PER1 proteins (Allingham et al., 1998; Caba et al., 2003; 2008). The activation of these nuclei are involved in several process related to food intake and to the synchronization of pups to this circadian signal (reviewed in Caba & González-Mariscal, 2009). Present results reinforce the proposal that periodic ingested milk is an entraining signal in the hypothalamus considering that gastrointestinal signals are relayed from gut to forebrain nuclei through brainstem structures.

In the rat, mechanical distention of the stomach by food activates vagal receptors, which, in turn, relay excitatory signals to gastric sensory subregions of the DVC (Rinaman et al., 1998). In rabbit pups, suckling of milk induces a strong stomach distention (Caba et al., 2003; Morgado et al., 2008; 2010). As shown in the results, 1.5 h after suckling milk there is a sharp increase in the expression of FOS in the NTS, DMX and AP, in agreement with studies of adult rats after meal ingestion and gastric distention (Fraser et al., 1995; Willing & Berthoud, 1997; Rinaman et al., 1998). At the same time we also found a high induction of FOS in PBN, similar to that seen in rats after ingestion of a large meal (Angeles-Castellanos et al., 2005). The lateral portion of PBN receives a dense projection from NTS (Herbert et al., 1990; Berthoud, 2002; Rinaman, 2010) and then PBN projects to several forebrain regions related to food intake control (Berthoud, 2002).

Several studies have revealed that not only the PBN but also the NTS send direct projections to hypothalamic nuclei related with satiety and with energy balance including the dorsomedial hypothalamic nucleus, arcuate nucleus, SON and PVN (Saper & Loewy, 1980; Thompson and Swanson, 1998; Renner et al., 2010; Rinaman, 2010). Although our results suggest that the PBN, but not the NTS, are functionally involved in integration of gastrointestinal information important for FAA (see below).

Although these neural pathways have not been characterized in the rabbit, our results suggest that these brain stem nuclei also play a role in activation of forebrain structures in this species. Previously, we found a significant increase of FOS in DMN, SON and PVN of neonatal rabbit pups after ingestion of a large volume of milk (Caba et al., 2008). Taken together, present and previous results in the rabbit pup agree with results in the rat suggesting the importance of brainstem nuclei activation after ingestion of a meal as well as the role of their projections to activate nuclei in the hypothalamus critical for control of food intake. Besides that, there is also a relationship with respect to FAA. In the rat entrained by food there is also an induction of FOS in NTS, AP and PBN as already mentioned (Angeles-

Castellanos et al., 2005) and the authors of this work suggest that this activation may provide essential entraining signal for the putative FEO. But while the NTS (Davidson, 2009) and AP (Davidson et al., 2001) are not necessary for FAA, as revealed by destruction of these nuclei in the rat, PBN and their projections may perhaps play a relevant role. Lesions of the lateral portion of the PBN result in a marked attenuation or complete loss of FAA (Davidson et al., 2000).

Of all the nuclei explored, the PBN is the only one that shows a similar tendency in the expression of PER1 in both RF02:00 and RF10:00 groups. Four hours after nursing there is a steady increase of PER1 that peaks 12 h after nursing in both groups, either nursed during the night or day. In other species several stimuli induces *mPer1* and their protein. It is well known that light induces *Per1* in the suprachiasmatic nucleus (Hamada et al., 2004), but this gene also can be induced by other cues. In vitro experiments in cultured rat-1 fibroblast cell line indicate that *mPer1* can be induced by several stimuli including serum shock, cAMP, protein kinase C, glucocorticoid hormones or Ca^{2+} (Balsalobre et al., 2000). Moreover PER1 can also be induced *in vivo*. In both the rodent and rabbit brain, PER1 rhythms are shifted and set by restricted feeding (Angeles-Castellanos et al., 2007; Feillet et al., 2008; Caba et al., 2008; Miñana-Solís et al., 2009; Morgado et al., 2011), and determination of phase of *Per1* gene and protein expression is commonly used as an index of synchronization by food.

When comparing PER1 oscillations of explored brain stem structures, the synchronization of PER1 rhythm in PBN supports the proposal that this nucleus is part of the putative FEO (Davidson et al., 2000), which now is considered to be comprised of a complex network that involves both central and peripheral structures (Angeles-Castellanos et al., 2005; Davidson 2009; rev. in Antle & Silver, 2009).

Unlike the synchronization of PER1 seen in the PBN, the DVC nuclei do not show a clear pattern of PER1 oscillations. In the rat, not all structures in the brain that express PER1 or PER2, show a clear rhythm, and even if they do, those rhythms are not always synchronized and entrained by a food pulse (Angeles-Castellanos et al., 2007; Feillet et al., 2008; Verwey et al., 2007; 2008). Nonetheless, the lack of synchronization of PER1 by nursing in AP and NTS in our results is striking considering the important role of these structures in transmitting metabolic and humoral signals to the brain. AP is a circumventricular organ that lacks blood-brain barrier and detects circulating humoral factors, and both the AP and NTS are key brain structures that receive afferent cues from the gastrointestinal system and their content. However destruction of both AP and NTS do not affect FAA in rats (Davidson et al., 2001; Davidson, 2009), but destruction of PBN affects FAA as already mentioned (Davidson et al., 2000).

Unlike the synchronization of rhythms observed during nursing, but in fasting FOS rhythm did not persist and PER1 rhythm shifted. In the rat, entrainment by food induces a rapid sharp increase of FOS in NTS, AP and PBN, which persists for several hours and declines around 8 h after food ingestion. However this increase does not persisted in fasted subjects (Angeles-Castellanos et al., 2005), similar to present results in the rabbit, and the rhythm of PER1 in PBN of nursed subjects shifted in fasted subjects as already mentioned. Unfortunately there are no reports of clock genes and their proteins in DVC and PBN in other species entrained by food to compare to our results. However, the lack of persistence of PER1 in fasted subjects suggests that this nucleus is important for synchronization only when food is present. At the same time, our results suggest that the PBN perhaps is also important in indicating the lack of food to other brain structures, considering that there is a significant elevation of PER1 in PBN, 1.5 h after expected time of nursing in fasted subjects previously nursed either during the night or the day. On this regard, lack of food is a

stressful signal for fasted rabbit pups as revealed by a significant increase of corticosterone (Rovirosa et al., 2005; Morgado et al., 2008, 2010). In the mouse, physical and inflammatory stressors produce a rapid induction of *mPer1* in the PVN, 1 h after application of stressor (Takahashi et al., 2001). So it is tempting to speculate that lack of food, due to an empty stomach or to an unidentified hormonal or metabolic cue during fasting induce PER1 in PBN of our subjects. Future studies should explore this possibility. In addition, examination and assessment of rhythms of other clock genes and their proteins, in the PBN and other brainstem areas, is warranted in future experiments in order to better understand the mechanisms underlying synchronization by food.

On basis of present and our previous work we conclude that DVC and PBN are part of the pathways conveying entraining signals to other parts of the brain to induce FAA in rabbit pups. Among these nuclei, PBN seems to also play an important role in the integration of food intake information perhaps as one of the components of the proposed distributed system of clocks that work in concert to produce FAA.

Acknowledgments

We gratefully acknowledge Biol. Mercedes Acosta for her invaluable help with maintaining and caring for the rabbit colony and to Dr. Michael N. Lehman for valuable comments and corrections on this manuscript. This research was partially supported by National Institutes of Health/Fogarty grant R01TW006636 (M. Caba).

References

- Allingham K, von Saldern C, Brennan PA, Distel H, Hudson R. Endogenous expression of c-Fos in hypothalamic nuclei of neonatal rabbits coincides with their circadian pattern of suckling-associated arousal. *Brain Res.* 1998; 783:210–218. [PubMed: 9507136]
- Antle MC, Silver R. Neural basis of timing and anticipatory behaviors. *Eur J Neurosci.* 2009; 30:1643–1649. [PubMed: 19878281]
- Angeles-Castellanos M, Aguilar-Roblero R, Escobar C. c-Fos expression in hypothalamic nuclei of food-entrained rats. *Am J Physiol Regul Integr Comp Physiol.* 2004; 286:R158–R165. [PubMed: 12933360]
- Angeles-Castellanos M, Mendoza J, Díaz-Muñoz M, Escobar C. Food entrainment modifies the c-Fos expression pattern in brain stem nuclei of rats. *Am J Physiol Regul Integr Comp Physiol.* 2005; 288:R678–R684. [PubMed: 15550615]
- Angeles-Castellanos M, Mendoza J, Escobar C. Restricted feeding schedules phase shift daily rhythms of c-Fos and protein Per1 immunoreactivity in corticolimbic regions in rats. *Neuroscience.* 2007; 144:344–355. [PubMed: 17045749]
- Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G, Schibler U. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science.* 2000; 289:2344–2347. [PubMed: 11009419]
- Berthoud HR. Multiple neural system controlling food intake and body weight. *Neurosci Biobehav Rev.* 2002; 26:393–428. [PubMed: 12204189]
- Caba M, Rovirosa MJ, Silver R. Suckling and genital stroking induces FOS expression in hypothalamic oxytocinergic neurons of rabbit pups. *Dev Brain Res.* 2003; 143:119–128. [PubMed: 12855183]
- Caba M, Tovar A, Silver R, Morgado E, Meza E, Zavaleta Y, Juárez C. Nature's food anticipatory experiment: entrainment of locomotor behavior, suprachiasmatic and dorsomedial hypothalamic nuclei by suckling in rabbit pups. *Eur J Neurosci.* 2008; 27:432–443. [PubMed: 18215239]
- Caba M, González-Mariscal G. The rabbit pup, a natural model of nursing-anticipatory activity. *Eur J Neurosci.* 2009; 30:1697–1706. [PubMed: 19863657]
- Conover WJ, Iman RL. Rank transformation as a bridge between parametric and nonparametric statistics. *Am Stat.* 1981; 35:132–133.

- Davidson AJ. Lesion studies targeting for food-anticipatory activity. *Eur J Neurosci.* 2009; 30:1658–16764. [PubMed: 19863659]
- Davidson AJ, Susan L, Cappendijk T, Stephan FK. Feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats. *Am J Physiol Regul Integr Comp Physiol.* 2000; 278:R1296–R1304. [PubMed: 10801300]
- Davidson AJ, Aragona BJ, Stephan FK. Persistence of meal entrained circadian rhythms following area postrema lesions in the rat. *Physiol Behav.* 2001; 74:349–354. [PubMed: 11714499]
- Davidson AJ, Poole A, Yamazaki S, Menaker M. Is the food- entrainable oscillator in the digestive system? *Genes Brain Behavior.* 2003; 2:1–8.
- Emond M, Schwartz GJ, Moran TH. Meal-related stimuli differentially induce c- Fos activation in the nucleus of the solitary tract. *Am J Physiol Regul Integr Comp Physiol.* 2001; 280:1315–1321.
- Feillet CA, Mendoza J, Albrecht U, Pévet P, Challet E. Forebrain oscillators ticking with different clock hands. *Mol Cel Neurosci.* 2008; 37:209–221.
- Fraser KA, Raizada E, Davison JS. Oral-pharyngeal-esophageal and gastric cues contribute to meal-induced c-fos expression. *Am J Physiol Regul Integr Comp Physiol.* 1995; 268:R223–R230.
- Hamada T, Antle MC, Silver R. Temporal and spatial expression patterns of canonical clock genes and clock-controlled genes in the suprachiasmatic nucleus. *Eur J Neurosci.* 2004; 19:1741–1748. [PubMed: 15078548]
- Herbert H, Moga M, Saper C. Connections of the parabrachial nucleus with the nucleus of the solitary tract and medullary reticular formation in the rat. *J Comp Neurol.* 1990; 293:540–580. [PubMed: 1691748]
- Hironaka S, Shirakawa T, Toki S, Kinoshita K, Oguchi H. Feeding-induced c-fos expression in the nucleus of the solitary tract and dorsal medullary reticular formation in neonatal rats. *Neurosci Lett.* 2000; 293:175–178. [PubMed: 11036189]
- Jilge B. The ontogeny of circadian rhythms in the rabbit. *J Biol Rhythms.* 1993; 8:247–260.
- Landry GJ, Kent BA, Patton DF, Jaholkowski M, Marchant EG, Mistlberger RE. Evidence for time-of-day dependent effect of neurotoxic dorsomedial hypothalamic lesions on food anticipatory circadian rhythms in rats. *PLoS One.* 2011; 6:e24187. [PubMed: 21912674]
- Messen, H.; Olszewski, JA. Cytoarchitectonic atlas of the rhombencephalon of the rabbits. New York, NY: 1949.
- Miñana-Solís MC, Angeles-Castellanos M, Feillet CA, Pévet P, Challet E, Escobar C, Mistlberger RE. Differential effects of a restricted feeding schedule on clock-gene expression in the hypothalamus of the rat. *Chronobiol Int.* 2009; 26:808–820. [PubMed: 19637044]
- Mistlberger RE. Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neurosci Biobehav Rev.* 1994; 18:171–195. [PubMed: 8058212]
- Morales T, Aguilar L, Ramos E, Mena F, Morgan C. Fos expression induced by milk ingestion in the caudal brainstem of neonatal rats. *Brain Res.* 2008; 1241:76–83. [PubMed: 18823956]
- Morgado E, Gordon MK, Miñana-Solis MC, Meza E, Levine S, Escobar C, Caba C. Hormonal and metabolic rhythms associated with the daily scheduled nursing in rabbit pups. *Am J Physiol Regul Integr Comp Physiol.* 2008; 295:R690–R695. [PubMed: 18480239]
- Morgado E, Meza E, Gordon MK, Pau KY, Juárez C, Caba M. Persistence of hormonal and metabolic rhythms during fasting in 7-to 9-day-old rabbits entrained by nursing during night. *Horm Behav.* 2010; 58:465–472. [PubMed: 20478309]
- Morgado E, Juárez C, Melo AI, Domínguez B, Lehman MN, Escobar C, Meza E, Caba M. Artificial feeding synchronizes behavioral, hormonal, metabolic and neural parameters in mother-deprived neonatal rabbit pups. *Eur J Neurosci.* 2011; 34:1807–1816. [PubMed: 22098455]
- Morgan JI, Curran T. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Ann Rev Neurosci.* 1991; 14:421–451. [PubMed: 1903243]
- Pau KY, Ma YJ, Yu JH, Yang SP, Airhart N, Spies HG. Topographic comparison of the expression of norepinephrine transporter, tyrosine hydroxylase and neuropeptide Y mRNA in association with dopamine beta-hydroxylase neurons in the rabbit brainstem. *Brain Res Mol Brain Res.* 1997; 48:367–381. [PubMed: 9332734]

- Renner E, Szabó-Meltzer KI, Puskás N, Tóth ZE, Dobolyi A, Palkovits M. Activation of neurons in the hypothalamic dorsomedial nucleus via hypothalamic projections of the nucleus of the solitary tract following refeeding of fasted rats. *Eur J Neurosci*. 2010; 31:302–314. [PubMed: 20074225]
- Rinaman L. Ontogeny of hypothalamic-hindbrain feeding control circuits. *Develop Psychobiol*. 2006; 48:389–396.
- Rinaman L. Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. *Brain Res*. 2010; 1350:18–34. [PubMed: 20353764]
- Rinaman L, Baker EA, Hoffman GE, Stricker EM, Verbalis JG. Medullary c-Fos activation in rats after ingestion of a satiating meal. *Am J Physiol Regul Integr Comp Physiol*. 1998; 275:262–268.
- Rogers, RC.; Hermann, GE. Central regulation of brainstem gastric vago-vagal control circuits. In: Ritter, S.; Ritter, RC.; Barnes, CD., editors. *Neuroanatomy and Physiology of Abdominal Vagal Afferents*. CRC Press; Boca Raton, FL: 1992. p. 99-134.
- Roviroso MJ, Levine S, Gordon MK, Caba M. Circadian rhythm of corticosterone secretion in the neonatal rabbit. *Dev Brain Res*. 2005; 8:92–96. [PubMed: 16026857]
- Stephan FK. The “other” circadian system: food as a zeitgeber. *J Biol Rhythms*. 2002; 17:284–292. [PubMed: 12164245]
- Saper CB, Loewy AD. Efferent connections of the parabrachial nucleus in the rat. *Brain Res*. 1980; 197:291–317. [PubMed: 7407557]
- Takahashi S, Yokota SI, Hara R, Kobayashi T, Akiyama M, Moriya T, Shibata S. Physical and inflammatory stressors elevate circadian clock gene *mPer1* levels in the paraventricular nucleus of the mouse. *Endocrinology*. 2001; 142:4910–4917. [PubMed: 11606459]
- Thompson RH, Swanson LW. Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. *Brain Res Rev*. 1998; 27:89–118. [PubMed: 9622601]
- Verwey M, Khoja Z, Stewart J, Amir S. Differential regulation of the expression of *Period2* protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats. *Neuroscience*. 2007; 147:277–285. [PubMed: 17544223]
- Verwey M, Khoja Z, Stewart J, Amir S. Region-specific modulation of *PER2* expression in the limbic forebrain and hypothalamus by nighttime restricted feeding in rats. *Neurosci Lett*. 2008; 440:54–58. [PubMed: 18541376]
- Willing AE, Berthoud HR. Gastric distention-induced c-fos expression in catecholaminergic neurons of rat dorsal vagal complex. *Am J Physiol Regul Integr Comp Physiol*. 1997; 272:R59–R67.

Figure 1.

Figure 2.

Figure 3.

Figure 4.