Food entrainment modifies the c-Fos expression pattern in brain stem nuclei of rats

Manuel Ángeles-Castellanos, Jorge Mendoza, Mauricio Díaz-Muñoz, and Carolina Escobar

Department of Anatomy, Facultad de Medicina and Instituto de Neurobiología, Juriquilla, Universidad Nacional Autónoma de México, Ciudad Universitaria, México

Submitted 30 August 2004; accepted in final form 10 November 2004

Food entrainment modifies the c-Fos expression pattern in brain stem nuclei of rats. Am J Physiol Regul Integr Comp Physiol 288: R678–R684, 2005. First published November 18, 2004; doi:10.1152/ajpregu.00590.2004.—When food is restricted to a few hours daily, animals increase locomotor activity 2–3 h before food access, which has been termed food anticipatory activity. Food entrainment has been linked to the expression of a circadian food-entrained oscillator (FEO) and the anatomic substrate of this oscillator seems to depend on diverse neural systems and peripheral organs. Previously, we have described a differential involvement of hypothalamic nuclei in the food-entrained process. For the food entrainment pathway, the communication between the gastrointestinal system and central nervous system is essential. The visceral sympathetic input to the brain stem arrives at the dorsal vagal complex and is transmitted directly from the nucleus of the solitary tract (NST) or via the parabrachial nucleus (PBN) to hypothalamic nuclei and other areas of the forebrain. The present study aims to characterize the response of brain stem structures in food entrainment. The expression of c-Fos immunoreactivity (c-Fos-IR) was used to identify neuronal activation. Present data show an increased c-Fos-IR following meal time in all brain stem nuclei studied. Food-entrained temporal patterns did not persist under fasting conditions, indicating a direct dependence on feeding-elicited signals for this activation. Because NST and PBN exhibited a different and increased response from that expected after a regular meal, we suggest that food entrainment promotes ingestive adaptations that lead to a modified activation in these brain stem nuclei, e.g., stomach distension. Neural information provided by these nuclei to the brain may provide the essential entraining signal for FEO.

Food-entrained c-Fos protein provides an increased c-Fos-IR signal in the paraventricular nucleus (PeF), and tuberomammillary nucleus. Food-entrained c-Fos-IR patterns persisted after 3 days of fasting in the DMH, LH, and PeF, suggesting an involvement of these nuclei in a time-keeping system entrained by feeding schedules. In contrast, we observed a marked cellular activation in the paraventricular nucleus (PVN) after meal time, which did not persist in fasting, suggesting that this structure is mainly involved with the response to signals elicited after feeding and could therefore be part of the entraining pathway. As a continuation of this study, we have now attempted to describe the role of brain stem nuclei as mediators of visceral input in a food-entrainment protocol. Previous studies have reported that visceral denervation due to subdiaphragmatic vagotomy or capsaiacin administration has no effect on food entrainment (9, 12, 28). However, we cannot rule out that in intact animals, these structures may be playing an important role by transmitting food-entraining signals.

The transmission of visceral information to the CNS relies mainly on brain stem nuclei. The visceral input to the brain stem arrives first to the dorsal vagal complex; this complex comprises the NST, the dorsal motor nucleus of the vagus (DMX), and the area postrema (AP) (5, 6). Afferents from the autonomic system providing information about the visceral state project primarily to the NST and AP. Because the AP can also sense circulating humoral signals, it is considered a chemosensitive portion of the vagal complex (25). The NST and AP project to the parabrachial nucleus (PBN), and the PBN and the NST provide visceral information to hypothalamic nuclei involved with the control of feeding behavior (4), such as the PVN, DMH, and LH, and to other areas of the forebrain.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
According to the model of FEO based on a distributed system, communication between the gastrointestinal system and the CNS is an essential element for food entrainment. A previous study by Davidson et al. (11) reported that bilateral lesions of PBN resulted in a marked attenuation of the FAA in food-entrained rats, but AP lesions did not alter FAA expression (10). Altogether, these findings suggested that food entrainment may rely mainly on neural peripheral signals and not on humoral signals entering through the AP. Thus the transmission of visceral information to the brain via brain stem nuclei could play an important role as entraining input elicited by feeding to the FEO.

The present study’s aim was to characterize the response of brain stem structures as input pathways in a food-entrainment protocol. This response was studied using c-Fos-IR as a marker of cellular activation (29). We hypothesized that cellular activation after meal time would point out structures involved with the reception or modulation of input visceral signals elicited by feeding, some of them possibly playing a role as entraining signals to the FEO. If this cellular activation is dependent on signals elicited by feeding, we would expect that this activation pattern would not be observed when interrupting this stimulus under total food deprivation.

MATERIALS AND METHODS

Subjects and housing conditions. Adult male Wistar rats, weighing 250–300 g, were maintained in a 12:12-h light-dark cycle (LD; lights on at 0700), regulated temperature (22 ± 1°C), free access to tap water and to regular laboratory rat diet (Rodent Laboratory Chow 5001), unless otherwise stated. Rats were acclimated to environmental conditions for at least 1 wk before starting the experimental procedures. Animal handling was conducted, according to the National Guide for Care and Use of Animal Experimentation (Decreto ley de protección a los animales del Distrito Federal, Gaceta Oficial del Distrito Federal, 26/02/02), which complies with the guiding principles for research of the American Physiological Society and those of the National Research Council (30).

Groups and food entrainment. The first stage of the experiment explored the c-Fos-IR in the brain stem during food entrainment. Rats were randomly assigned to one of four feeding conditions for 3 wk and were housed in groups of the same condition in transparent acrylic cages (40 × 50 × 20 cm). The ad libitum group (AL; n = 36) had free access to food; this group allowed us to observe the expected c-Fos-IR under regular feeding conditions in a LD cycle. The food-restricted group (RF; n = 36) was maintained under a restricted feeding schedule with food available daily for 2 h (meal time, 1200) and was defined as food-zeitgeber time 0 (F-ZT0). To discard the possibility that observed effects could be due to either a 22-h food deprivation interval or the fasting-refeeding conditions, two additional control groups were included (each n = 6). For a 22-h fasting control (22F), rats were maintained with free food access for 3 wk. On the last day of the experiment, food was removed at 1400 and after 22 h of food deprivation. At F-ZT0, rats were perfused. Other rats (22F-R) were left 22 h in fasting, refeed for 2 h, and then perfused and processed at F-ZT2 for immunohistochemistry.

The second stage of the experiment explored the persistence of the entrained c-Fos-IR pattern in fasting. Rats were assigned to one of two groups: one group was food entrained for 3 wk, as the RF group, then allowed free food access for 5 days and left food-deprived for 3 days (RF-AF). This procedure assessed the persistence of food-entrained rhythms for an 8-day interval. A fasting control group (Fast) was allowed free food access for 3 wk and then was left food-deprived for 3 days.

On the last day of each feeding protocol, rats were perfused and brains were obtained. It has been estimated that the intracellular cascade of events can produce peak levels of expression of the protein Fos in ~45 min (29, 39), and with that in mind, tissue was labeled (and is presented here) according to the approximate corresponding
time of the releasing stimulus. Brains were obtained for the time points 1000, 1100, 1200, 1400, 1800, and 2000; sampling points are further defined according to meal time as F-ZT22, F-ZT23, F-ZT0, F-ZT2, F-ZT6, and F-ZT8, respectively (n = 6 per group and time). On the day of perfusion, animals of the F-ZT0 time point in the RF group remained unfed until anesthetized and processed.

Histology and immunohistochemistry. Rats were anesthetized with an overdose of pentobarbital sodium (Sedal-Vet 65 mg/ml) and were perfused transcardially, with 250 ml of 0.9% saline followed by 250 ml of fixative PLP (4% paraformaldehyde, 1.4% lysine, 0.2% sodium m-periodate) in PBS (0.1 M, pH 7.2). Brains were removed, postfixed for 1 h in PLP, and cryoprotected in 10, 20, and 30% sucrose for 24 h, respectively.

Brains were frozen and cut at 18°C in horizontal sections of 40 μm. Data corresponding to the hypothalamus of these same rats were already reported in a previous paper (2). Sections were serially collected in four sets, one set was stained with cresyl violet acetate (Sigma Chemical), and a second set was processed for immunohistochemistry. Brains were then blocked using a vector kit (Vector Labs, Burlingame, CA), and sections were incubated with polyclonal antibodies against c-fos (1:100) or c-jun (1:100) (StressGen, Victoria, Canada) and visualized using a horseradish peroxidase-antiperoxidase (HRP-AP) kit (Amersham, Arlington Heights, IL). Immunoreactive cells were visualized using a light microscope (Nikon Optiphot-2) and counted by an operator (M.C.C.) who was blind to the experimental conditions. All counts were performed in the 3 subdivisions of the NST: rostral, medial, and caudal and in the PBN lateral and medial and in the AP and DMX. The number of immunoreactive cells (IR) in the hypothalamus (HA) was also counted in the same rats. Results are presented as the mean ± SE.

Table 1. Statistical values obtained with the two-way ANOVA for independent measures, for the group entrained to feeding schedules and its ad libitum controls and the six time points

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Group</th>
<th>Time</th>
<th>Group × Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F (1,60)</td>
<td>P &lt;</td>
<td>F (5,60)</td>
</tr>
<tr>
<td>NST</td>
<td>Rostral</td>
<td>23.2</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>157.3</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Caudal</td>
<td>73.4</td>
<td>0.001</td>
</tr>
<tr>
<td>PBN</td>
<td>Lateral</td>
<td>27.6</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>7.81</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>AP</td>
<td>17.7</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>DMX</td>
<td>20.9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

NST, nucleus of the solitary tract; PBN, parabrachial nucleus; AP, area postrema; DMX, dorsal motor nucleus of the vagus; NS, not significant.
chemistry for c-Fos as follows: The sections were incubated for 72 h at 4°C in primary antibody (rabbit anti-Fos; Santa Cruz Biotechnology) diluted 1:2500 in PBS, 1% goat serum, and 0.03% Triton X-100 (PBSGT). Tissue was then incubated in biotinylated secondary antibody (goat anti-rabbit; Vector Laboratories) 1:200 in PBSGT for 2 h at room temperature, followed by incubation in avidin-biotin complex (0.9% avidin and 0.9% biotin solutions; Vector Laboratories) in PBSGT for 2 h at room temperature. Tissue was then reacted in diaminobenzidine (0.5 mg/ml, in Trizma buffer 7.2) with hydrogen peroxide (35 μl, 30% H₂O₂). Between each step, tissue was rinsed three times, for 10 min in PBS. Tissue was mounted on gelatin-coated slides, and label was intensified with osmium tetroxide 0.1% for 30 s. (Baker Analyzed), dehydrated with alcohol, cleared with xylene, and cover slipped with Permount.

Cell count. To quantify c-Fos expression in brain stem nuclei, two representative sections were selected in accordance with the stereotaxic atlas from Paxinos and Watson (32). A first ventral section (Interaural 1.90 mm) was selected to quantify c-Fos-IR in the NST, AP, and DMX. A second section (Interaural 2.66 mm) contained the PBN. For analysis, NST was divided in medial, caudal, and rostral sections, according to Ter Horst and Streefland (36). Also, the PBN was divided in medial and lateral subregions for analysis (20). Images of the corresponding cresyl violet sections were acquired to identify and define the sampling area for each nucleus. Images of selected sections were obtained using a computerized image analysis system (Meta Vue series 4.5, Universal Imaging) attached to a Nikon light microscope (Nikon Eclipse E600). On the left side of each section, a grid of 25 squares 40 μm was superimposed on the center of the selected structure, and c-Fos-IR cells located in the area of the grid were counted at a 10× magnification. To minimize the number of false positives, the background optic density was established in a nearby region lacking c-Fos-IR; stained cells that reached or surpassed three times the background optic density were considered positive and were included, whereas cells under this staining threshold were discarded. A single examiner, who was blinded to treatment conditions, performed all counts.

Data analysis. The number of cells for each sampled area are expressed as cells per millimeters squared. Data were classified by groups and time and are represented as means ± SE. Data between the AL and RF group, as well as data for RF-AF vs. Fast, were compared with a two-way ANOVA for independent measures with a factor for group (2 levels) and a factor for time (6 levels). Values from the 22F and the 22F-R group were compared with values of the RF group, at F-ZT0 and at F-ZT2, respectively, using a one-way ANOVA for independent measures. The two-way ANOVAs were followed by Tukey’s post hoc test with significant values set at P < 0.01. Statistical analysis was performed with the program Statistica version 4.5 (StatSoft, 1993).

RESULTS

All nuclei studied in the brain stem exhibited c-Fos-IR. The AL group exhibited constant counts in all time points and no evident oscillations. In the RF rats a significant increase of c-Fos-IR was observed in all brain stem nuclei after feeding in F-ZT2 and F-ZT6, as can be observed for the NST (Fig. 1). The two-way ANOVA showed a significant effect for all nuclei because of the factor group, the factor time, and their interaction (Table 1). In the three regions of the NST (Fig. 2) there was no difference in the number of c-Fos-IR cells at F-ZT0 between RF group and the 22F group, but after feeding at F-ZT2, there was a significant difference in the number of c-Fos-IR cells between RF and 22F-R [rostral F(1,8) = 13.3; P < 0.001; medial F(1,8) = 100.9; P < 0.001; caudal F(1,8) = 22.7; P < 0.001].
In the PBN, in both lateral and medial, RF rats showed an increase in c-Fos-IR at ZT2 and ZT6 in response to feeding (Fig. 3). There was no difference in the number of c-Fos-IR cells between F-ZT0 of RF group and the 22F group, and after meal time at F-ZT2, both PBN regions in the RF group were different from the 22F-R group \[\text{medial, } F(1,8) = 35.8; P < 0.001; \text{lateral, } F(1,8) = 9.8; P < 0.05\].

In the AP and the DMX (Fig. 4), the number of c-Fos-IR cells at F-ZT0 and F-ZT2 in the RF group were not different from the 22F group and the 22F-R, respectively.

c-Fos-IR in fasted rats. The fasted groups exhibited low c-Fos-IR expression in all nuclei compared with the AL and the RF groups, perhaps because of the catabolic state caused by 3 days of fasting. The food-entrained pattern observed previously in the RF group did not persist in the RF-FA group. In addition, this latter group did not show a difference in c-Fos-IR cells from the Fast group (Figs. 2B, 3B, 4B). ANOVA showed no effect due to factor group, time, or their interaction (Table 2).

**DISCUSSION**

Present results show that during food-entrainment there is an increased c-Fos-IR after feeding (F-ZT2 and F-ZT6) in all evaluated brain stem structures. This response was statistically different from the AL group for all nuclei. Before meal time, none of the nuclei exhibited different values from the AL or 22F groups. Thus main effects were only expressed after feeding. In addition, values in the NST and PBN at F-ZT2 were different from the expected response after a fasting-refeeding process (22F-R). Because the food-entrained pattern did not persist in fasting conditions, we assume that the c-Fos IR activation in all of these nuclei is promoted by signals elicited by feeding and/or digestion. These results are consistent with previous data, which have reported increased c-Fos-IR in brain stem nuclei after a meal (15, 31).

The NST is the main afferent relay structure receiving visceral information from the periphery and transmitting it to the CNS. The caudal subregion receives, among others, afferents from the stomach, gut, and the liver. Previous studies have reported increased c-Fos expression in NST due to stomach distension produced by inflation of a gastric balloon (18) or by feeding (14). Therefore, we assume that the c-Fos-IR activation

Table 2. Statistical values obtained with the two-way ANOVA for independent measures for the refeeding-fasting group and the fasted controls

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Group</th>
<th>Time</th>
<th>Group × Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F (1,54) P&lt;</td>
<td>F (5,54) P&lt;</td>
<td>F (5,54) P&lt;</td>
</tr>
<tr>
<td>NST</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rostral</td>
<td>1.02 NS</td>
<td>2.63 NS</td>
<td>3.34 NS</td>
</tr>
<tr>
<td>Medial</td>
<td>0.27 NS</td>
<td>0.37 NS</td>
<td>1.85 NS</td>
</tr>
<tr>
<td>Caudal</td>
<td>0.12 NS</td>
<td>1.88 NS</td>
<td>1.69 NS</td>
</tr>
<tr>
<td>PBN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral</td>
<td>2.00 NS</td>
<td>0.50 NS</td>
<td>0.99 NS</td>
</tr>
<tr>
<td>Medial</td>
<td>5.81 NS</td>
<td>1.36 NS</td>
<td>1.27 NS</td>
</tr>
<tr>
<td>AP</td>
<td>12.5 NS</td>
<td>3.80 NS</td>
<td>0.90 NS</td>
</tr>
<tr>
<td>DMX</td>
<td>25.4 0.01</td>
<td>3.37 NS</td>
<td>0.51 NS</td>
</tr>
</tbody>
</table>
observed at F-ZT2 and at F-ZT6 is mainly due to the stomach distension after feeding. In our study, c-Fos-IR at F-ZT2 and at F-ZT6 in the RF group was different from that expected after a regular meal, compared with the 22F-R group. The observed cellular activation suggests that signals elicited in food-entrained rats were different and more intense than those produced by visceral activation after a regular meal. In a previous study, we reported that RF rats develop an enhanced capacity of stomach distension that reaches 200% of the stomachs of AL animals after a regular meal. Also, the stomach of RF rats exhibits a slow emptying rate that allows food absorption for a prolonged interval (24). This adaptive process was also observed in young rabbits, which are nursed only once a day by their lactating dams (17). This indicates that NST plays an important role receiving specific signals elicited during food entrainment that are transmitted to the brain and possibly to the food-entrained system.

In the NST rostral region there was a decrease in activity at F-ZT6. The rostral region of NST receives afferents from the glossopharyngeal, facial, vagal, and hypoglossal nerves, providing sensitive and motor information of the mouth and tongue (5). Thus in this region the decrease in c-Fos-IR at F-ZT6 in RF animals may reflect a reduction of the oropharyngeal signals evoked by feeding behavior.

The c-Fos-IR in the AP increased after meal time at F-ZT2 and F-ZT6 with a similar pattern as that observed in the NST; however, in this area the response at F-ZT2 in RF rats was not different from the expected response after a regular feeding episode as observed in the 22F-R group. This suggests that the signals arriving at the AP in RF rats are of similar nature as those elicited after a regular meal. The AP is an organ that lacks a blood-brain barrier and responds mainly to humoral signals from the periphery such as CCK, but not to the mechanical distension of the stomach by a gastric balloon (18, 19). This strongly suggests that under RF, humoral signals after feeding are similar to those elicited by a regular feeding event. A previous study did not find any effects on food entrainment in rats with AP lesions (11), which points to the relevance of neural signals provided by other structures in entraining the FEO.

In the PBN the c-Fos-IR in RF rats was different from the expected response after a 22-h fasting, 2-h refeeding event. The PBN is a second-order relay nucleus for neural projections from the NST and AP to the brain (4, 20). Thus the c-Fos-IR activation in RF rats was probably due to information of stomach distension and oropharyngeal signals from the NST. The pattern of c-Fos-IR was similar in both lateral and medial PBN regions, and both receive the same afferents from NST and AP (20). A previous study reported that bilateral ibotenic acid lesions of PBN produced a decrease in FAA onset and intensity. The authors concluded that because PBN is a relay structure from the periphery to the hypothalamus and other regions of the brain, lesions were partially eliminating the incoming signal to entrain the FEO (11). Our results indicate that under food entrainment, the PBN exhibits a different and enhanced response to a meal, and thus confirm that PBN may be a relevant structure for the food-entrainment pathway.

The DMX is the main parasympathetic output to the periphery; it receives direct afferents from the paraventricular hypothalamic nucleus (7), which also exhibits increased c-Fos-IR in RF rats after meal time (1, 2, 8). Information from the PVN and NST projects to the DMX (7, 33) and then to the digestive system through the vagal nerve to produce responses for initiation of gastrointestinal motor and secretory events. The response in the digestive tract promotes multiple neural and humoral feedback signals that contribute to the correct coordination of the events associated with digestion. In this study, the response in the DMX in RF rats did not differ from the expected response before and after a regular meal in the control 22F and 22F-R groups.

Our results indicate that brain stem nuclei involved in the transmission of digestive and oropharyngeal information to the brain are activated after meal time during a food-entrainment paradigm. They also indicate that under food entrainment, the NST and PBN exhibit a different increased c-Fos-IR response from that observed after a single fasting-refeeding event. This differential response may be due to modified adaptive adjustments of the stomach or of other digestive organs. We cannot discard the possibility that other signals associated with meal time can also provide entraining signals for FEO. Previous studies have reported that corticosterone (3), epinephrine (37), and insulin (22) can entrain peripheral oscillators and can produce FAA (26). Thus hormonal changes may also provide time cues to central or peripheral oscillators. However, our data point to a relevant activation in brain stem nuclei, especially NST and PBN, in phase with meal time, which may be important elements to transmit specific signals involved with food entrainment.

ACKNOWLEDGMENTS

We thank Dr. Raúl Aguilar (Instituto de Fisiología Celular, Universidad Nacional Autónoma de México) for providing a cryostat to cut the sections described in this study. We also thank Dr. Horacio de la Iglesia (Department of Neurology, University of Massachusetts) for comments and grammatical corrections to this manuscript.

GRANTS

This study was supported by grants CONACyT 43950-M and Dirección General de Asuntos de Personal Académico IN-203803, IX-227504–1.

REFERENCES


