Circadian rhythms in mammals are predominantly entrained to the day-night cycle, but under restricted feeding schedules (RFS) several rhythms are entrained to mealtime (34). When food is restricted to a few hours daily, animals develop in a circadian range, free runs for several cycles during constant-fasting conditions, and exhibit transients in response to phase shifts of food access (34). The suprachiasmatic nucleus in the hypothalamus (SCN) has been identified as the light-entrained oscillator (LEO), whereas the mechanisms underlying the food-entrained oscillator (FEO) remain elusive. Food entrainment depends on a circadian oscillator independent of the SCN, because FAA is elicited in animals with bilateral lesions of the SCN (33). In an attempt to characterize the FEO, several groups have produced lesions in potential anatomic substrates of this oscillator and explored its effect on the expression of FAA. In food-entrained rats, bilateral lesions of the ventromedial hypothalamic nucleus (VMH) did not prevent FAA or the preceding corticosterone peak (19, 24). Likewise, bilateral lesions of the lateral nucleus (LH) and the paraventricular nucleus (PVN) failed to abolish FAA (23), and similar negative results were obtained after lesion of the limbic system (26) and brain stem areas (9, 10). However, a recent study described that RFS induced phase control of mPer rhythmicity in the cerebral cortex and hippocampus, suggesting a possible link of both structures with FAA (37).

Recent studies have pointed out the importance of peripheral oscillators as potential components of FEO (8, 40) and have directed special attention to the liver as a possible peripheral clock. However, we reported that rats made cirrhotic with a chronic CCl4 treatment exhibit FAA that persisted in fasting conditions (17), indicating that the expression of FAA does not depend on an intact liver. Similar effects were reported in rats made diabetic with a single dose of streptozotocine (11), discarding the relevance of the pancreas and insulin secretion as part of this oscillator.

Because the identification of a single structure as substrate of FEO has proven unsuccessful, it has been suggested that the FEO may be constituted by a distributed and possibly redundant system of peripheral organs interacting with central nervous structures involved with ingestive behavior and energy balance (15, 34). However, the possible elements of this oscillator, associated with either the input of the entraining signals, the time-keeping mechanisms, or rhythmicity transmission, remain to be identified.

The immunohistochemical expression of c-Fos-like proteins in the nervous system is considered a marker of neuronal activation (28). The logic of this methodology is based on the demonstration that the expression of this protein is increased in neurons after extracellular electrical or chemical stimulation (28). Thus c-Fos-like expression has been extensively used to identify neurons activated by experimental treatments, including metabolic events and food ingestion (2, 14, 36).

The present study was aimed to identify the hypothalamic nuclei involved with food entrainment by using c-Fos immunoreactivity (c-Fos-IR) as a marker of functional activation. In a first stage, we characterized the temporal profile of c-Fos-IR in the hypothalamus of food-entrained rats [restricted feeding (RF)] and their ad libitum controls (AL), and in a second stage we explored whether temporal patterns of c-Fos-IR observed in food-entrained rats persisted during fasting. We hypothesized...
that a significant increase in c-Fos-IR for time points preceding food access would point out structures involved with the expression of FAA, as part of either the clock’s mechanisms or its output pathway. An increase in c-Fos-IR after food ingestion would point out structures responding to food-related cues and thus be involved with the entraining pathway of the FEO. For the persistence group, we hypothesized that in fasting conditions a significant increase in c-Fos-IR during the expected mealtime would point out constituents of an endogenous time-keeping mechanism or of its output pathway.

MATERIALS AND METHODS

Subjects and housing conditions. Adult male Wistar rats weighing 250–300 g were obtained from the general bietorium of the Medical Faculty in the Universidad Nacional Autónoma de México. Animals were maintained in a 12:12-h light-dark cycle (LD: lights on at 0700 = Zeitgeber time 0 (ZT0)), with regulated temperature (22 ± 1°C), free access to tap water, and 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.

Groups and food entrainment. A first stage of the experiment explored the c-Fos-IR associated with 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 AL group had free access to food; this group allowed for the observation of the expected c-Fos-IR under regular feeding conditions. The RF group was maintained under RFS with food available daily for 2 h (1200–1400, ZT5–ZT7). To determine that observed effects were not due to either an acute 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 (ZT7), and after 22 h of food deprivation (at 1200, ZT5) six rats were perfused. Another six rats (22F-R) were left 22 h in fasting and then refed for 2 h (ZT5-ZT7) and were then perfused and processed for immunohistochemistry.

The second stage of the experiment explored the persistence of the entrained c-Fos-IR pattern after 3 days in fasting conditions. Rats were assigned to one of two groups. The persistence group (RF-F) was food entrained for 3 wk similar to the RF group, then allowed free food access for 5 days, and left food deprived for 3 days (food was removed at ZT7). This procedure allowed us to assess the persistence of FAA for an 8-day interval and allowed us to discard the expression of an hourglass clock. A fasting control group (Fast) was allowed free food access for 3 wk and then was left food deprived for 3 days.

For all groups, rats were perfused and brains were obtained on the last day of each feeding protocol at ZT3, ZT4, ZT5, ZT7, ZT11, or ZT13 (n = 6 per group and time). In addition, to obtain a circadian curve for the SCN as a positive control, additional rats from the AL and RF groups were perfused at ZT17 and ZT23.

Because it is estimated that the intracellular cascade of events to produce peak levels of the protein c-Fos takes ~45 min (38), tissue was labeled and data are presented according to the approximate releasing stimulus time. The day of perfusion, animals of the ZT5 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 4% paraformaldehyde, 1.4% lysine, and 0.2% sodium m-periodate (PLP) 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.

Brains were frozen and cut at −18°C in horizontal sections of 40 μm to have complete slides of the brain, including the brain stem (to be used in another study). Sections were serially collected in four sets. One set was stained with cresyl violet acetate (Nissl), and a second set was processed for immunohistochemistry for c-Fos. The sections were incubated for 72 h at 4°C in primary antibody (rabbit anti-Fos) diluted 1:2,500 in PBS, 1% goat serum, and 0.3% Triton X-100 (PBSTG). Tissue was then incubated in biotinylated secondary antibody (goat anti-rabbit; Vector Laboratories) 1:200 in PBSTG for 2 h at room temperature, followed by incubation in avidin-biotin complex (0.9% avidin and 0.9% biotin solutions; Vector Laboratories) in PBSTG 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 placed under a coverslip with Permount.

Cell count. To quantify c-Fos expression in hypothalamic nuclei, four representative sections were selected in accordance with the stereotaxic atlas from Paxinos and Watson (30). A first ventral section (interaural 0.78 mm) was selected to quantify c-Fos-IR in the SCN. A second section (interaural 0.90 mm) contained the VMH, LH, the dorsomedial nuclei (DMH), and the tuberomammillary nuclei (TM). A third section (interaural 1.18) contained the perifornical area of the lateral hypothalamus (PeF); and a more dorsal section (interaural 1.90 mm) was used to sample the PVN. Images of the corresponding Nissl sections were acquired to identify and define the area for each nucleus. Images of the immunohistochemical preparation were obtained using a computerized image analysis system (MCID Image Analyzer Imaging Research) attached to an Olympus light microscope (BHT). A grid of 4 × 4, 40-μm squares (1,600 μ㎡) was superimposed on the center of each nucleus, and only c-Fos-IR cells in the left side of each section were manually counted at a ×10 magnification. To minimize the number of false positives, background optic density (OD) was established in a nearby region lacking c-Fos-IR. When the observer marked a c-Fos-IR cell, the program indicated its OD, and stained cells that reached or surpassed three times the background OD were considered positive and were counted, whereas cells under this staining threshold were not considered. The examiner who performed all counts was not aware of the treatment received by the individual animals.

Data analysis. Number of cells for each sampled area were transformed to cells per square millimeter. 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-F 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). In addition, a one-way ANOVA was used to determine significant oscillations in the temporal pattern (6 levels) in each group. All ANOVAs were followed by a Tukey post hoc test with significant values set at P < 0.01. Values from the 22F and the 22F-R group were compared with the ZT5 and the ZT7 values of the RF group, respectively, with a one-way ANOVA for independent measures. Statistical analysis was performed with Statistica version 4.5 (StatSoft, 1993).

RESULTS

c-Fos-IR associated with RFS. A clear c-Fos-IR was observed in all hypothalamic nuclei here reported (see Fig. 1). In the AL group, DMH, LH, PVN, VMH, and PeF did not show significant fluctuations throughout time in the number of c-Fos-IR cells (Figs. 2A, 3A, 4A, 5A, and 6A); however, effects of sampling time were observed in the TM (Fig. 7A) and SCN (Fig. 8A). The SCN exhibited a diurnal rhythm adjusted to the
LD cycle, with higher values during the early morning and lowest values after lights off, whereas the TM showed a decrease of c-Fos-IR cells at ZT4 and a gradual increase toward the night. The one-way ANOVA revealed only significant effects due to time in the TM and in the SCN ($P < 0.01$; Table 1).

RFS imposed a different c-Fos-IR temporal pattern in several hypothalamic nuclei compared with AL. The DMH, LH, PeF, and TM nuclei showed an increase in the number of c-Fos-IR cells 1 h before food access and a remarkably increased c-Fos-IR after meal time (Figs. 2A, 3A, 6A, and 7A). The PVN showed a marked increase in c-Fos-IR cells but only immediately after feeding (Fig. 4A). In the TM, a second increase of activity was observed after lights off. In contrast, the VMH and the SCN did not show a different c-Fos-IR pattern between the RF and the AL (Figs. 5A and 8A). In the SCN, RF rats exhibited a c-Fos-IR pattern linked with the LD cycle. The one-way ANOVA revealed a significant effect of time in the DMH, PeF, PVN, and SCN (Table 1). The two-way ANOVA evidenced a significant main effect between the AL and RF groups in all nuclei; a significant main effect due to variations in time in the DMH, PeF, PVN, TM, and SCN; and a significant group-time interaction in the DMH, PeF, and PVN (Table 2).

Because RFS impose a cycle of 2-h feeding/22-h fasting, data from the RF group at ZT5 and ZT7 were compared with the 22F and 22 F-R groups, respectively. The statistical comparison revealed a significant difference between 22F and RFS.
by higher c-Fos-IR cell counts during the light phase and a decrease after lights off (Fig. 8B); however, it was distorted due to the influence of 48 h in fasting. The one-way ANOVA only indicated a significant main effect due to time in the PeF (Table 1).

The RF-F group exhibited in all nuclei, with the exception of the SCN and VMH, which displayed a different c-Fos-IR temporal pattern from that observed in the Fast group, with increased values at the expected meal time. For the RF-F group, the one-way ANOVA revealed a significant time effect in the LH, PeF, and PVN (Table 1). In addition, for all nuclei but the SCN, the two-way ANOVA showed a significant difference between the RF-F and Fast groups, a significant time variation in the DMH and PeF, and a significant group-time interaction in the LH and PeF (Table 3).

**DISCUSSION**

Present data indicate that several hypothalamic nuclei exhibit a specific c-Fos-IR temporal pattern associated with RFS and may be involved with the FEO’s mechanisms. They also indicate that, specifically in the SCN and the VMH, the
temporal pattern of cellular activity remains phase tight to the LD cycle and is not modified due to RFS. Thus SCN and VMH may not be involved with the FEO’s mechanisms.

Our initial hypothesis proposed that hypothalamic structures would respond with a differential pattern, depending on their involvement with specific mechanisms of FEO. Structures involved with FAA expression would show increased c-Fos-IR at time points preceding food access. If involved with the clock’s mechanism, the activation pattern would persist in fasting conditions. An increase in c-Fos-IR after food ingestion would point out structures responding to food-related cues, and we would thus assume their role in the FEO’s entraining pathway. However, data in this study point out a more complex pattern and do not allow for the assumption of such deterministic conclusions.

Food entrainment produced increased c-Fos-IR in the DMH, LH, PVN, TM, and PeF in anticipation of food access, and in the same structures an even stronger activation was observed after mealtime. Cellular activation due to RFS was different from that observed in AL, 22F, and 22FR groups, which confirmed that this activation was specific for the food-entrained condition. In addition, in nearly all nuclei, increased values were observed during fasting around the expected mealtime, but this was only significant for the LH and PeF, and for the LH, the temporal pattern was even more evident than that observed during RFS. To better understand how the present structures can be linked to the FEO, these data must be examined according to the known role of each nucleus in metabolic balance and feeding behavior.

In the DMH, a slight increase in c-Fos-IR was observed in anticipation of mealtime and a significant increase after food intake. This pattern of activation persisted in fasting conditions, although with lower intensity. The DMH is considered an intermediary structure among hypothalamic nuclei of the medial zone due to its intrahypothalamic afferent and efferent projections (3), and because of the high levels of glucose receptors it is regarded as an integrator of metabolic functions (4). In addition, anatomic tracers have shown first-order synaptic projections between the SCN and DMH (13), suggesting that it may be an important output pathway for the transmission of circadian rhythmicity. Present data indicate that under RFS the DMH uncouples from the SCN and exhibits a pattern of cellular activation phase related to the mealtime. A similar pattern as in the DMH was observed in the LH, but in this structure the persistence response was significantly enhanced. Kurumiya and Kawamura (22) reported food entrainment of multiunitary activity in the LH, which persisted for several cycles in fasting, suggesting an important relationship between the LH and the FEO. In addition, the LH is distinguished by abundance of fibers and cell bodies that project to the cerebral cortex, the brain stem, and autonomic cells in the spinal cord, and thus is considered an integrative structure that transmits energy
balance information to extrahypothalamic sites (23). Because both structures, DMH and LH, play an important integrative role in transmitting and processing metabolic signals and feeding behavior, and taking into account present evidence, we suggest that both may be elements of the FEO’s clock mechanisms or that they may be elements of the clock’s output.

Another relevant effect observed in this study was the intense activation of the TM and PeF during the hours before and after food access as well as during persistence. Both structures are located in the posterior hypothalamus and have been related to a behavioral arousal system (18, 39). The PeF of the lateral hypothalamus was recently described as an important arousal-promoting region containing orexigenic neurons (39) that project widely to forebrain and brain stem regions (27). Due to the location of orexin receptors, this system is thought to regulate sleep-wake cycles, locomotor activity, and energy homeostasis and feeding behavior (31, 39). In addition, a previous study (20) described an intense neuronal activation in the TM related to the arousal state in rats anticipating one of two daily meals. Our data are in agreement with this study, and we may speculate that an increase of c-Fos-IR in the TM and PeF may be related to the arousal state in rats during anticipation, during feeding, and while expecting food access in fasting.

The SCN and the VMH did not show significant cellular activation in association with food entrainment. Our data are in agreement with previous studies that have described entrainment by feeding schedules of the clock gene per1 mRNA in peripheral tissues and central nervous structures but no phase control in the SCN (8, 37). Also, it has been well documented that animals with a bilateral lesion of the SCN are still able to entrain to feeding schedules and exhibit FAA (33). However, it is important to point out that RFS rats exhibited higher c-Fos-IR values than AL rats, which indicates a modulation of SCN activity by feeding schedules as previously described (7). Although, the VMH is a hypothalamic structure that has been linked to feeding behavior, in the literature we find evidence that extensive lesions of the VMH do not disrupt or hinder either metabolic food entrainment or the display of FAA (19, 24). As previously described, Kurumiya and Kanawamura (22) reported entrainment of the temporal pattern of electrical multiunitary activity in the VMH and lateral LH in food-entrained rats. This food-entrained pattern persisted in the LH up to 4 days in total food deprivation (constant conditions), but dampened in the VMH. Present data are in agreement with previous findings and confirm that the VMH and SCN are not elements of FEO.

Significant and intense cellular activation was observed in the PVN after food intake in food-entrained rats. This response
did not persist in fasting conditions, although during the expected mealtime a slight increase of c-Fos-IR was observed and this pattern was statistically different from the control Fast group. Previous studies indicate that the PVN integrates the information of stomach fill and the availability of substrates and represents an important output pathway to the autonomic nervous system, modulating digestive motor functions, gastrointestinal secretion, and pancreatic function via oxytocinergic projections to the nucleus of the solitary tract and the dorsal motor nucleus of the vagus (5, 32). Gastric load and distension elicit cellular activation in the PVN observed through c-Fos expression, norepinephrine, and oxytocin release (6, 29, 35). We have observed that food-entrained rats (unpublished data) and young rabbits (16) develop an exceptional capacity to distend their stomach, and this stomach distension coincides with increased c-Fos IR in the PVN (2, 6). Thus we can suggest that the intense c-Fos-IR response observed in the RF group was due to stomach distension after food ingestion, and the lower c-Fos-IR observed in the RF-F group corresponds to persistence of water intake, as previously described (17). As a result, it is possible that this gut-PVN axis may play an important role in the FEO’s entraining pathway.

The fact that a previous study producing bilateral lesions of PVN and LH was unable to disrupt FAA expression (25) gives support to a multistructural model of the FEO built up by several nuclei in the central nervous system. This is further supported by a study that reported oscillating clock genes in several hypothalamic nuclei (1) and a study that demonstrated independence of clock gene’s rhythmicity in the periphery from FAA expression. This latter study evidenced that behavioral food entrainment relies on central mechanisms and not on a peripheral clock (12).

Because present data are based on the sampling of a single horizontal section for each nucleus, its interpretation must be addressed with limitations, especially for those nuclei that contain many neuronal phenotypes, although this study provides evidence that several hypothalamic nuclei exhibit cellular activation patterns related and specific to food entrainment. The specific c-Fos-IR patterns under RFS and in fasting suggest that the PVN may be involved with the entraining pathway, the PeF and TM with the arousal state, and the DMH and LH with the clock’s mechanisms of FEO or its output. In addition, present data

### Table 1. One-way ANOVA F values for the main effect of time in AL and RF, RF-F and Fast groups

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Group</th>
<th>AL F&lt;sub&gt;S,28&lt;/sub&gt;</th>
<th>RF F&lt;sub&gt;S,28&lt;/sub&gt;</th>
<th>RF-F F&lt;sub&gt;S,28&lt;/sub&gt;</th>
<th>Fast F&lt;sub&gt;S,28&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>DMH</td>
<td>AL</td>
<td>0.55</td>
<td>12.3†</td>
<td>2.41</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>RF-F</td>
<td>2.09</td>
<td>26.7†</td>
<td>3.39†</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>PVN</td>
<td>5.16†</td>
<td>2.03</td>
<td>1.56</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>VMH</td>
<td>1.76</td>
<td>0.86</td>
<td>2.51</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>SCN</td>
<td>F&lt;sub&gt;S,34&lt;/sub&gt; = 8.96†</td>
<td>F&lt;sub&gt;S,34&lt;/sub&gt; = 3.81†</td>
<td>1.06</td>
<td>2.41</td>
</tr>
</tbody>
</table>

AL, ad libitum; RF, restricted feeding; RF-F, RF-fasting; Fast, fasting; DMH, dorsomedial nucleus; LH, lateral nucleus; PeF, perifornical area; PVN, paraventricular nucleus; TM, tuberomammilar nucleus; VMH, ventromedial nucleus; SCN, suprachiasmatic nucleus. *P < 0.05; †P < 0.01.

### Table 2. F values obtained with the two-way ANOVA for independent measures, for the group entrained to feeding schedules, and its ad libitum controls

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Group</th>
<th>Time F&lt;sub&gt;S,58&lt;/sub&gt;</th>
<th>G × T F&lt;sub&gt;S,58&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMH</td>
<td>AL</td>
<td>44.75†</td>
<td>8.12†</td>
</tr>
<tr>
<td></td>
<td>RF-F</td>
<td>29.37†</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>PVN</td>
<td>12.00†</td>
<td>9.38†</td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>12.73†</td>
<td>4.31†</td>
</tr>
<tr>
<td></td>
<td>VMH</td>
<td>8.05†</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>SCN</td>
<td>F&lt;sub&gt;S,68&lt;/sub&gt; = 3.17</td>
<td>F&lt;sub&gt;S,68&lt;/sub&gt; = 8.09†</td>
</tr>
</tbody>
</table>

G × T, group-time interaction. *P < 0.05; †P < 0.001.

### Table 3. F values obtained with the two-way ANOVA for independent measures, for the RF-F group and its fasted controls

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Group</th>
<th>Time F&lt;sub&gt;S,58&lt;/sub&gt;</th>
<th>G × T F&lt;sub&gt;S,58&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>DMH</td>
<td>AL</td>
<td>39.15†</td>
<td>3.62*</td>
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</tr>
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<td>PVN</td>
<td>12.58†</td>
<td>3.66*</td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>9.20*</td>
<td>1.81</td>
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<tr>
<td></td>
<td>VMH</td>
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<tr>
<td></td>
<td>SCN</td>
<td>0.01</td>
<td>1.75</td>
</tr>
</tbody>
</table>

*P < 0.05; †P < 0.001.
indicate that the FEO may be a distributed system of interacting structures. Further characterization of the FEO’s mechanisms will need an integrative approach in which other extrahypothalamic structures are taken into account.

GRANTS
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