The infralimbic cortical area commands the behavioral and vegetative arousal during appetitive behavior in the rat

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Abstract

The infralimbic cortical area is a good candidate to send processed motivational signals to initiate the arousing and autonomic responses that characterize appetitive behaviors. To test this hypothesis we enticed hungry rats with food while assessing locomotion (as an index of arousal level) and temperature responses, and evaluated Fos immunoreactivity (IR) in the infralimbic area and in subcortical nuclei involved in thermoregulation or arousal. We also recorded from single infralimbic neurons in freely moving rats while enticing them with food. We found that 83% of infralimbic neurons were excited or inhibited by feeding and, in particular, that 33% of infralimbic neurons increased their discharge rate during food enticing. Intact rats showed increased Fos IR in the infralimbic area, as well as in many other cortical areas. The excitotoxic lesion of the infralimbic cortex abolished the arousing and hyperthermic responses observed in intact rats, as well as the expression of Fos IR in the ascending arousal system and subcortical thermoregulatory regions. We conclude that the infralimbic area plays a central role in implementing behavioral arousing and thermal responses during an appetitive behavior.

Introduction

Motivation imparts direction and intensity to behavior (Hebb, 1955). The direction is set by the actions to obtain a reward or to avoid punishment, and the intensity is provided by the increased arousal that characterizes the appetitive phase of a motivated behavior. Arousal has been operationally defined as increased sensory, motor and emotional responsiveness (Pfaff *et al.*, 2002), and will be assessed here by measuring locomotor activity, as well as by observing the rats' behavior. We have previously shown (Valdés *et al.*, 2005) that food presentation to hungry rats increased arousal evaluated by polysomnographic recordings. Hebb (1955) emphasized the need to consider the cortical feedback to the recently discovered nonspecific projection system (or arousal system), and GoldmanRakic (1987) made the point that the prefrontal cortex is the only cortical source of feedback connections to the arousal system, and so it is the way for the cortex to control its own activity. The appetitive phase of motivated behaviors has distinctive preparatory physiological changes, such as increases in behavioral arousal (Pfaff *et al.*, 2002) and core temperature (Valdés *et al.*, 2005).

Growing evidence supports the idea that the medial prefrontal cortex, and particularly the infralimbic area (IL), is a key structure in controlling the behavioral and neural responses to aversive or stressful stimuli (Milad & Quirk, 2002; Morgan *et al.*, 2003; Amat *et al.*, 2005). We have shown (Recabarren *et al.*, 2005) that IL lesions prevent the anticipatory rise in temperature shown by rats under a restricted feeding schedule, where food is available for a few fixed hours each day. This anticipatory rise in temperature is thought to be under the control of a food-entrainable circadian oscillator (Mistlberger, 1994). We have also found, using an experimental paradigm not involving food-related circadian signals, that food enticing induces a significant increase in core temperature in hungry but not in satiated rats (Valdés *et al.*, 2005). Based on our previous studies, we hypothesized that the infralimbic cortex is involved not only in the control of aversive responses but also in the appetitive responses to food enticing, probably by means of the direct excitatory connections of the infralimbic cortex to the ascending arousal system neurons and to subcortical neurons controlling temperature.

To test the idea that the IL may be involved in feeding-related behavioral and vegetative arousal induced by food presentation, we evaluated Fos immunoreactivity (IR) in the prefrontal cortex or made single unit recordings from the IL in freely moving rats enticed by food. To evaluate the importance of the IL in behavioral and autonomic arousal induced by food enticing, we studied the effect of bilateral IL excitotoxic lesions on locomotor activity and thermal responses, respectively, as well as the changes in Fos IR in the ascending arousal system and in subcortical regions involved in thermal responses.

Materials and methods

Subjects

We used 56 male adult Sprague-Dawley rats from the institutional Animal Care Facility, weighing 250–350 g. They were individually housed and had permanent access to water and food pellets, except when indicated. The experimental protocols included environmental temperature controlled at 23 °C. All experiments were carried out in accordance with the National Institute of Health (USA) Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996). The institutional Bio Safety and Ethical Committee approved these experimental protocols, which minimized the number of rats used.

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Telemetric transponder implantation and infralimbic cortex lesions

Rats were anesthetized with intraperitoneal injections of 100 mg/kg of ketamine (Imalgene™, Rhodia Merieux, Santiago de Chile, Chile) plus 20 mg/kg of xilazine (Rompun™, Bayer, Santiago de Chile, Chile). One PDT-4000 E-mitter sensor (MiniMitter, Sun River, OR, USA) was implanted into the peritoneal cavity, under sterile conditions. Antibiotics were administered at the end of surgery (Enrofloxacin 5%, 19 mg/kg i.p., Bayer) together with a single dose of anti-inflammatory ketoprofen 1% (Ketophen, 0.2 mg/kg i.p., Rhodia Merieux). After surgery, the rats were moved to a recording room with a 12/12-h light/dark photoperiod (lights on at 07:00 h). The rats were allowed 1 week of recovery before initiating telemetric recordings. Locomotor activity and body temperature were measured with VITALVIEW (MiniMitter) software and hardware.

In the same surgical session, eight rats were lesioned in the IL. The rats were placed in a stereotaxic frame, following standard procedures (Paxinos & Watson, 1998). A single hole was drilled on the frontal bone to access the IL. Ibotenic acid (15 nL, 15 μg/μL, Sigma Chemical Co., St Louis, MO, USA) was pressure injected via glass micropipettes (10–15 μm tip) by pulses of air provided by a Pneumatic PicoPump model PV800 (World Precision Instruments, New Haven, CT, USA) at the following coordinates: 3.0 mm rostral to bregma, ±0.6 mm from midline and 4.8 mm in depth measured from the duramater (Paxinos & Watson, 1998).

Electrode implantation and electrophysiological recordings

Under ketamine/xilazine anesthesia (see above) and sterile conditions, three adult rats were implanted with a micromanipulator containing six independently movable tetrodes, three for each side of the brain. During 3 days following surgery, the rats received daily i.p. doses of antibiotic and analgesic, as above. Tetrodes were made of four twisted, 12-μm nichrome wires (H.P. Reid Co., Palm Coast, FL, USA), with 1–2 MΩ impedance. The tetrode electrode configuration greatly improved the yield and reliability of spike identification (Gray *et al.*, 1995). The tetrodes were lowered through plastic guiding tubes that were glued together in a circular array, spaced with a center-to-center distance of *c.* 200 μm. The distance between the centres of the left and right arrays was 1 mm. Five stainless steel screws were anchored to the skull to help secure the implant with dental acrylic; two of them were used as ground leads.

A small craniotomy was performed over the IL, at the same stereotaxic coordinates as used to lesion the IL (see above). An incision was made in the duramater and the guiding tube array was lowered into the cortex up to 1 mm above the target area. Each tetrode was then independently lowered to the target area, at a speed of 300 μm/20 min in each daily session, until appropriate signals could be recorded; it usually took 4 days to reach the IL. We recorded from 24 sites from the three rats, and each rat was subjected to one recording session/week for 4 weeks, with a mean yield of 3.5 neurons/site. The leads of the tetrodes were connected to a multichannel head stage and amplifier (LM324, National Semiconductor), and the signals were amplified (10 k), band pass filtered (0.5–5 kHz) and digitized at a rate of 27 kHz/channel using custom software. The resulting signals were passed through off-line sorting software to reconstruct the spike trains. For each of the different data sets, spike separation was achieved by an interactive custom-made computer program (McNaughton *et al.*, 1983; Gray *et al.*, 1995; Maldonado & Gray, 1996).

Experimental protocol

Locomotor activity and body temperature were continuously recorded while the rats were subjected to a protocol of 1 day of food *ad libitum*, 1 day of fasting (hungry rats, $n = 21$) or food *ad libitum* (satiated rats, $n = 5$) and 1 day of food presentation**.** Placing a closed wire-mesh box full of food pellets on top of the rat cage, for 30 min ($n = 5$), 60 min ($n = 5$) or 2 h ($n = 11$), induced the appetitive phase of feeding behavior. The wire box measured 10 cm on each side and the size of the mesh squares was 0.4 cm, so that the rats could smell, touch and see the food but could not get it out of the box or gnaw the pellets through the mesh. Three additional groups of rats were used as controls for arousal that was independent of food-seeking behavior. One group consisted of five naive rats under baseline conditions of *ad libitum* feeding; this group was used to assess basal Fos IR between 10:00 and 12:00 h. The second control group (forced wakefulness, $n = 4$) corresponded to hungry rats that were kept awake for 1 h, by gently shaking the rat cage every 5 min. A third group (empty box, $n = 5$) was presented with an empty and clean food box. To control for the Fos IR that is related to the act of eating, we studied a group of five rats that had food for 2 h after a 24-h fast.

Histology

At the end of the experiments, lesioned and intact rats were deeply anesthetized with 7% chloral hydrate (350 mg/kg, i.p.) and perfused through the left ventricle with a saline flush (100 mL) followed by 500 mL of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The brains were postfixed in the same fixative for 2 h and transferred to 30% sucrose with 0.02% sodium azide in PBS until they sank. Brains were cut frozen under dry ice in the coronal plane, at 50 μm thickness, using a sliding microtome.

Immunohistochemistry

We obtained three alternate series of sections from each brain. One series was stained with cresyl violet and the other two were used for immunohistochemistry. Free-floating sections were incubated in 0.3% H_2O_2 in PBS for 30 min, rinsed in PBS and transferred to the blocking solution (0.4% Triton-X100, 0.02% sodium azide, 3% normal goat serum in PBS) for 1 h. The sections were then transferred to the primary antibody incubation solution and left there overnight at room temperature (22–24 °C). This incubation solution contained the Fos polyclonal antibody (Ab-5, rabbit polyclonal, Oncogene, San Diego, CA, USA) diluted 1 : 20 000 in the blocking solution. The sections were rinsed in PBS for 1 h before being incubated in the secondary antibody solution [Biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (H + L), Jackson Immuno-Research, West Grove, PA, USA; diluted 1 : 1000 in 0.4% Triton X100, 1.5% normal goat serum in PBS]. After rinsing for 40 min, the sections were incubated for 1 h in Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 500 in PBS, rinsed and incubated in a 0.05% 3-3′ diaminobenzidine hydrochloride solution containing 0.003% H_2O_2, and 0.05% nickel chloride to obtain a dark blue reaction product. Selected sections from both series, already immunostained for nickel-enhanced Fos IR, were subjected to a second immunostaining to identify histaminergic, adenosine deaminase-immunoreactive neurons in the tuberomammillary nucleus (Senba *et al.*, 1985), tyrosine hydroxylase-immunoreactive neurons in the ventral tegmental area or locus coeruleus, or orexin-immunoreactive neurons in the lateral hypothalamic/perifornical area. The second immunostaining was performed after an overnight rinse in PBS with 0.02% sodium azide

and was revealed with 3-3′ diaminobenzidine hydrochloride with no nickel intensification, which yielded a brown cytoplasmic precipitate that contrasted with the dark violet nuclear 3-3′ diaminobenzidine hydrochloride-nickel labeling of the Fos IR. The antisera used were anti-adenosine deaminase (polyclonal, raised in rabbit, diluted 1 : 10 000, Chemicon, Temecula, CA, USA), anti-tyrosine hydroxylase (rabbit polyclonal, 1 : 10 000, Chemicon) and anti-orexin A (rabbit polyclonal, 1 : 2000, Phoenix Pharmaceutical Inc., CA, USA).

The specificity of the antibodies used has been tested by preadsorption of the antisera with the respective antigens: orexin A (Chen *et al.*, 1999), adenosine deaminase (Gerashchenko *et al.*, 2001), c-Fos (Constandil *et al.*, 1995) and tyrosine hydroxylase (Strack *et al.*, 1989). We also checked and confirmed the published distribution of the different antigens in brain tissue.

Sections stained with cresyl violet were used to determine the extent of the lesion. Camera Lucida drawings of the relevant sections, which included cortical areas and lesion boundaries, were superimposed over a flat map of the medial aspect of the forebrain, as previously described (Recabarren *et al.*, 2005). We traced on this map the bilateral extent of the lesions for each rat. We expressed the size of a particular lesion area in mm^2 and as the percentage of lesion area relative to the total bilateral surface of the corresponding cortical area. The cytoarchitectonic borders and nomenclature were based on the atlas of Swanson (1998).

Quantification of Fos immunoreactivity

The activation of the different nuclei of the ascending arousal system, thermoregulatory nuclei and IL was assessed by counting Fos-immunoreactive neurons bilaterally in two or three coronal sections from each nucleus per rat. In the tuberomammillary nucleus, lateral hypothalamic area and ventral tegmental area we counted neurons double labeled for Fos IR and either adenosine deaminase, orexin or tyrosine hydroxylase, respectively. For the locus coeruleus, laterodorsal tegmental nucleus, substantia innominata and dorsal raphe we used grids of appropriate size to count Fos-immunoreactive neurons, as reported in detail previously (Valdés *et al.*, 2005).

Thermoregulatory nuclei

In the preoptic area we used a horizontal 600 × 800-μm rectangle (0.48 mm^2), centered on the curvature adjacent to the supraoptic nucleus. Two sections were used, a rostral section (level −0.46), where the third ventricle is not divided, and a caudal section 50 μm apart just where the third ventricle is divided by the anterior commissure but is not fused with the lateral ventricle.

For the raphe pallidus we used a vertical 100 × 380-μm rectangle (0.038 mm^2), centered on the midline between the pyramids to count in three sections. The intermediate section (level −11.58) was at the rostral tip of the solitary nucleus. One rostral section (level −10.35) was identified by the caudal tip of locus coeruleus. The caudal section (level −14.16) coincided with the rostral end of the area postrema.

For the dorsomedial hypothalamic nucleus we used a 500-μm square (0.25 mm^2). The counting square was placed just lateral to the superior half of the third ventricle. Two sections were used (levels −2.85 and −3.25), corresponding to a level where the medial tips of the optic tract and cerebral peduncle are vertically aligned.

For the ventromedial hypothalamic nucleus we used a 500-μm square (0.25 mm^2) positioned lateral to the inferior half of the third ventricle, above the median eminence. Two sections were counted, a rostral section (level −2.45) and a caudal section (level −2.85).

For the IL we used a vertical 500 × 750-μm rectangle (0.375 mm^2) positioned just above the rhinal incisure, excluding lamina 1. We counted Fos-immunoreactive neurons in a rostral section (level +3.2) with the rostral tip of the forceps anterior to the corpus callosum, and a caudal section (level +2.8) where the forceps anterior is not contiguous to the cingulum bundle.

Statistical analysis was performed using SIGMASTAT 3.0 (SPSS, Chicago, IL, USA). The number of Fos-immunoreactive neurons in the different nuclei and under different treatments, locomotor activity in different animal groups and firing rate of neurons in different treatments were analysed by the Kruskal–Wallis one-way ANOVA, followed by the Dunn's multiple pair-wise comparison method. Temporal changes in body core temperature were analysed by two-way repeated measures ANOVA, followed by the Holm-Sidak multiple comparison method. We considered a difference as significant when $P < 0.05$. Data are expressed as mean ± SEM.

Results

We had previously shown that when hungry rats are enticed with food, they become behaviorally aroused while trying to get the food and have significant increases in core temperature averaging 0.6 °C (Valdés *et al.*, 2005). We used two approaches to evaluate the activation of the IL during food presentation: first, we quantified the number of Fos-immunoreactive neurons under different feeding-related conditions and second, we performed single unit recordings to directly measure neuronal activity in freely moving rats, under the same conditions.

Activation of the infralimbic area in intact rats during feeding behavior assessed by Fos immunoreactivity

In 33 rats we compared the number of Fos-immunoreactive neurons in this cortical area in six different conditions: (i) a 'hungry' group consisting of nine rats (in two of 11 rats we lost the sections from the rostral prefrontal cortex) killed after 2 h of being exposed to unreachable food; (ii) a 'satiated' group consisting of five rats fed *ad libitum* and killed after 2 h of food presentation; (iii) hungry rats presented with an empty wire box ($n = 5$); (iv) rats kept forcedly awake for 1 h ($n = 4$) and (v) hungry rats that had access to food for 2 h, the postprandial group ($n = 5$). (vi) The baseline number of Fos-immunoreactive neurons in the IL was obtained from a group of five naive rats that were killed at the same time of day (10:00–12:00 h) as the experimental groups.

We found a significant increase in the number of Fos-immunoreactive neurons in the IL during food presentation in the hungry group, as well as in those hungry rats that were allowed to feed (postprandial group; Fig. 1). Fasting *per se* or food enticing to satiated rats did not increase Fos IR in the IL above baseline levels. Similarly, rats kept forcedly awake for 1 h by tapping their cages showed no increase in Fos IR. Nonetheless, the increase in Fos IR during enticing or after eating was not restricted to the IL. Many other cortical regions also had increased Fos IR (Fig. 1d).

Activation of the infralimbic area during feeding behavior assessed by extracellular single unit recordings

As we have found increased Fos IR in the IL, we wanted to examine the actual discharge properties of cells during similar sets of feeding-related conditions. We obtained single unit recordings from 85 cells in three rats. The histological reconstruction of the electrode tracks (Fig. 2a) indicated that nearly 80% of the neurons were from the IL and the remaining neurons were from the medial orbital area (see

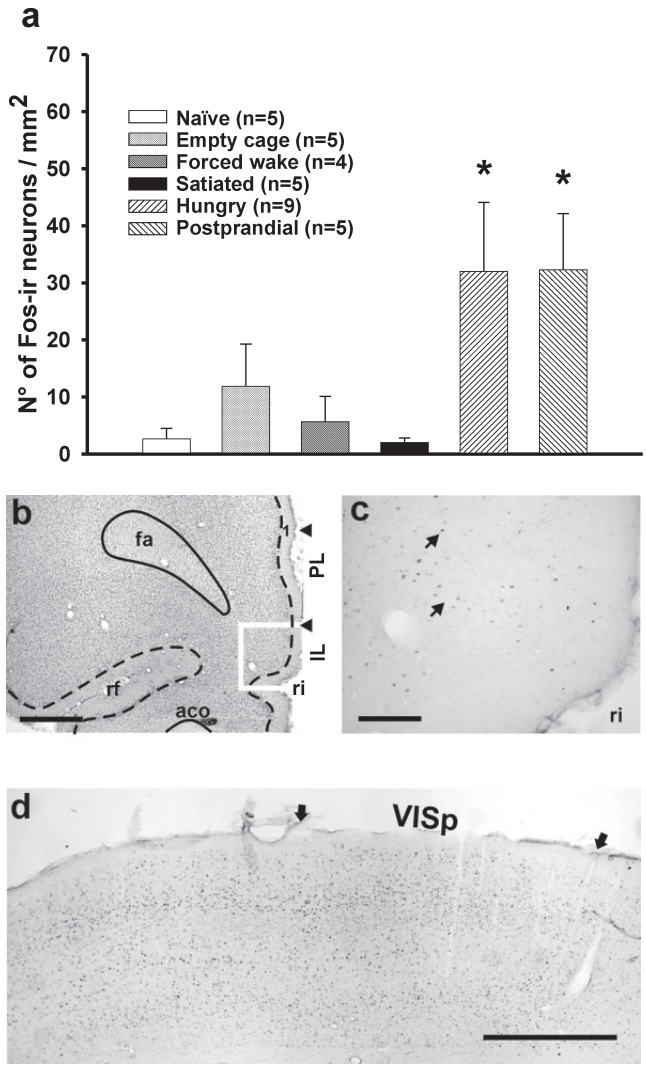

FIG. 1. Increased Fos immunoreactivity (IR) in the cortex of hungry rats during the appetitive phase of feeding. (a) Activation of the infralimbic area (IL) assessed by Fos IR in rats under different feeding-related experimental situations. *Significant differences from the naive condition. (b) Nissl-stained section through the medial prefrontal cortex. (c) Fos IR in the IL region within the rectangle in b; adjacent sections. (d) Fos IR in the primary visual cortex (VISp) and neighboring cortical areas. Scale bars, 500 μm (b and d); 100 μm (c). aco, anterior commissure, olfactory limb; fa, corpus callosum anterior forceps; PL, prelimbic area; ri, rhinal incisure; rf, rhinal fissure.

Table 1). The histogram of spontaneous firing rate of these 85 neurons followed a distinct, nonoverlapping bimodal distribution (Fig. 2f) with one population ($n = 64$; L neurons) firing at frequencies below 3.0 Hz and the other firing above 3.5 Hz ($n = 21$; H neurons). Each population had a normal distribution of spontaneous firing rates (Kolmogorov-Smirnov test, $P > 0.05$). Moreover, we found that the neurons recorded from rats under *ad libitum* (55 neurons) or under fasting (30 neurons) conditions exhibited similar firing rate distributions (Mann–Whitney test). The low firing rate population had a mean spontaneous discharge of 0.97 ± 0.08 Hz, whereas the faster firing rate population fired at an average of 7.29 ± 0.77 Hz. Thirty neurons (Table 1) recorded from hungry rats exhibited long-term stability, attested by a consistent spike shape, as judged by our cluster analysis (Fig. 2b and c). In this way, we were able to compare their discharge properties under four different consecutive feeding-related conditions during the daytime following a 24-h fast. The conditions were similar (except for the duration) to those described in the previous paragraph, i.e. fasting, a 10-min presentation of an empty food box, a 10-min enticing with a box full of rat pellets and, last, after eating those pellets. Satiated rats were not subjected to the same protocols, so we cannot tell whether fewer IL neurons would be active in response to those protocols. The 30 neurons from the hungry rats were continuously recorded for 10 min under each condition, allowing 15 min for eating the pellets after food presentation, and then recorded for another 10 min (Fig. 2d and e). We used ANOVA followed by multiple comparison Dunn's test to determine in which condition single neurons had a significantly higher or lower discharge rate relative to the other conditions. Remarkably, 83.3% (25 of 30) of the neurons changed their discharge rate during food-related conditions (Fig. 2g and Table 1), 10% were excited by the empty box and only two of 30 neurons were unresponsive. These numbers indicate that most IL neurons were sensitive to the food conditions. We observed that 46.7% (14 of 30) of the neurons increased their discharge in response to presentation and/or the postprandial condition. Interestingly, almost all of them (13 of 14) belonged to the population exhibiting a low spontaneous firing rate. Conversely, 36.7% (11 of 30) of the cells were suppressed by one or more food conditions, and they mostly belonged (seven of 11) to the group with higher spontaneous rates. An example of a low firing rate IL neuron (neuron 3) sensitive to food presentation is shown in Fig. 2d and e. Note that this cell, as well as all neurons with a similar response profile, maintained the increased discharge rate during food presentation. Although the tetrode technique allows for the simultaneous recording of neighboring cells, we found no tendency for nearby neurons to show similar responses.

Effects of infralimbic area lesions on behavioral and vegetative arousal

The activation of the IL, evaluated by Fos IR or by single unit recording, only shows that IL neurons modulate their activity during food presentation, but does not indicate whether this cortical area is specifically involved in the behavioral or vegetative responses. To

FIG. 2. Specific responses of single infralimbic neurons to different feeding-related conditions in freely moving rats. (a) Reconstruction of a recording track through the infralimbic area (IL). The arrow points to the electrolytic lesion in the dorsal taenia tecta (TTd) and the asterisk indicates one recording site in the IL. Bar, 500 μm. (b) An example of multiple single-unit recording with a tetrode. Each point in the plot represents an action potential that exceeded twice the noise threshold. The *x* and y axes represent peak amplitude of action potential recorded by channels 2 and 3, respectively. Upper panel, first 10 min of recording; lower panel, last 10 min. (c) The five neurons isolated by the cluster analysis during the first (upper panel) and last 10 min of recording from channels 1–4 (from left to right). (d) The firing rates of neurons 1, 2 and 3 during the 10 min of recording under the four different feeding conditions. The vertical dashed lines indicate the nonrecording periods between the conditions, which were of 15 min between presentation and postprandial and of 1 min between the other conditions. (e) The mean firing rate for neurons 1, 2 and 3, showing the significant increase (*$P < 0.05$, one-way ANOVA followed by multiple comparisons, Dunn's method) in firing rate of neuron 2 in the postprandial condition and of neuron 3 during food presentation relative to all other conditions. (f) Distribution of spontaneous firing rates for the 85 neurons recorded from the medial prefrontal cortex. Inset: histogram where neurons were classified into two groups. The H neurons had firing rates of 3.5 Hz and the L group fired at 3.0 Hz. The Kolmogorov-Smirnov test for normality suggested a bimodal distribution. (g) Grouping of neurons according to their responses to the experimental conditions (further details in Table 1) shows that most neurons were sensitive (excited or inhibited) to food conditions (presentation and postprandial). aco, anterior commissure, olfactory limb; fa, corpus callosum anterior forceps; PL, prelimbic area; ri, rhinal incisure.

evaluate the relevance of the IL in the food-related responses described below, as well as in the activation of arousal-promoting nuclei, or in the activation of subcortical regions controlling the thermal responses, we performed IL lesions and studied their effect on those variables. Bilateral ibotenic acid microinjections caused a loss of neurons and a marked gliosis (Fig. 3a and b) mostly involving the IL and sparing subcortical regions. The destruction ranged from 87.3% (Fig. 3a and c) to 23.9% (Fig. 3b and d) of the IL. The adjacent cortical areas were damaged to a much lesser extent (Table 2).

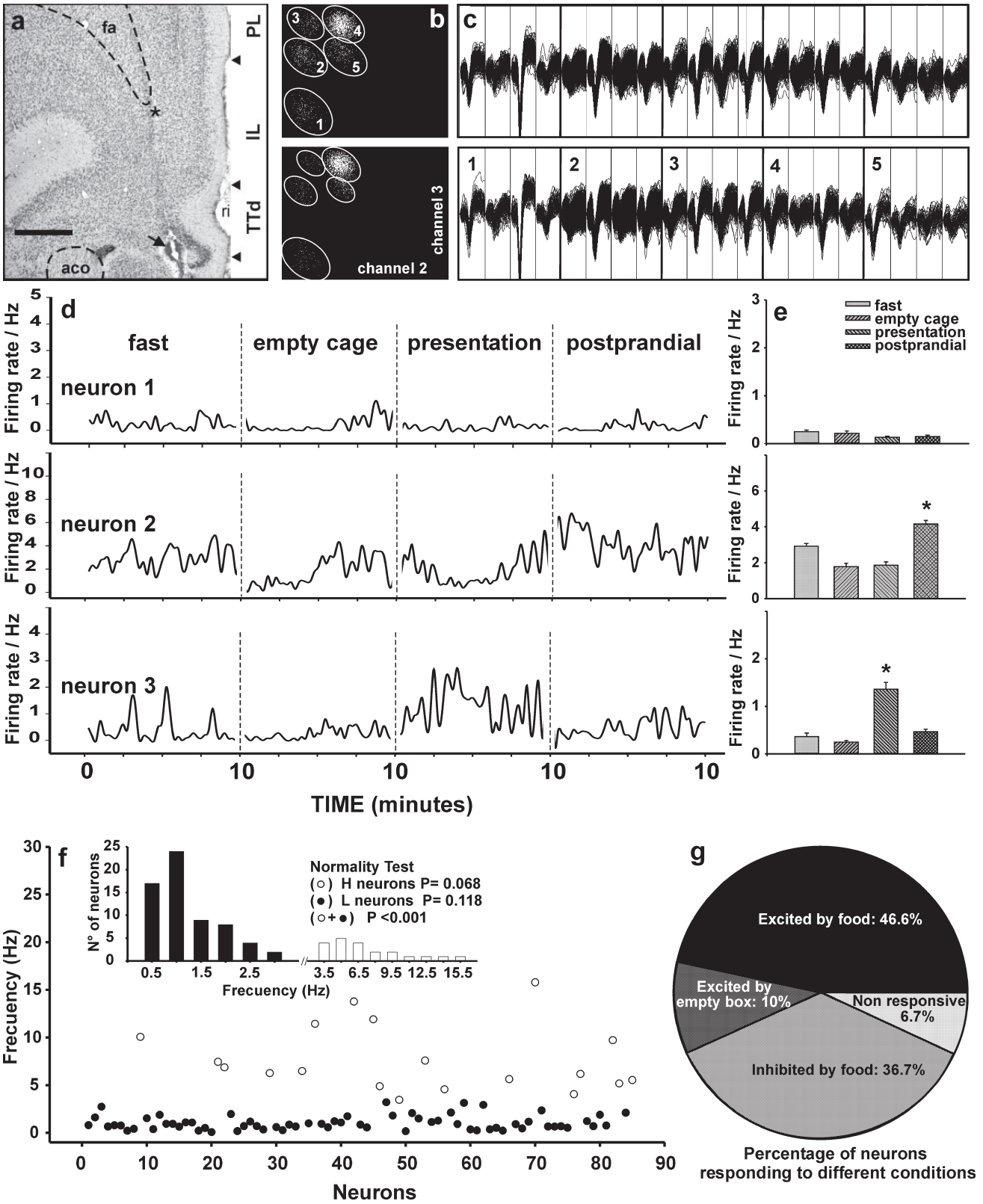

TABLE 1. Response selectivity of medial prefrontal cortex neurons

Type of response	Selective responses (%)	Selective responses (*n*/total)	Numbers of neurons with high and low basal firing rates: Infralimbic area (IL)	Numbers of neurons with high and low basal firing rates: Medial orbital area (ORBm)
Excited by empty box	10	(3/30)	1 high	2 low
Excited by food presentation	33.3	(10/30)	8 low, 1 high	1 low
Postprandial excitation	10	(3/30)	3 low	
Excited by food presentation and postprandial	3.3	(1/30)	1 low	
Inhibited during postprandial	6.7	(2/30)	1 low, 1 high	
Inhibited under the three conditions	20	(6/30)	2 low, 2 high	2 high
Inhibited by food presentation and postprandial	6.7	(2/30)	1 low, 1 high	
Inhibited by food presentation and empty box	3.3	(1/30)	1 high	
Nonresponsive	6.7	(2/30)	2 low	

Recordings were made for 10 min under each condition: presentation of an empty wire box, presentation of a wire box full of rat food and 15 min after eating part of the food.

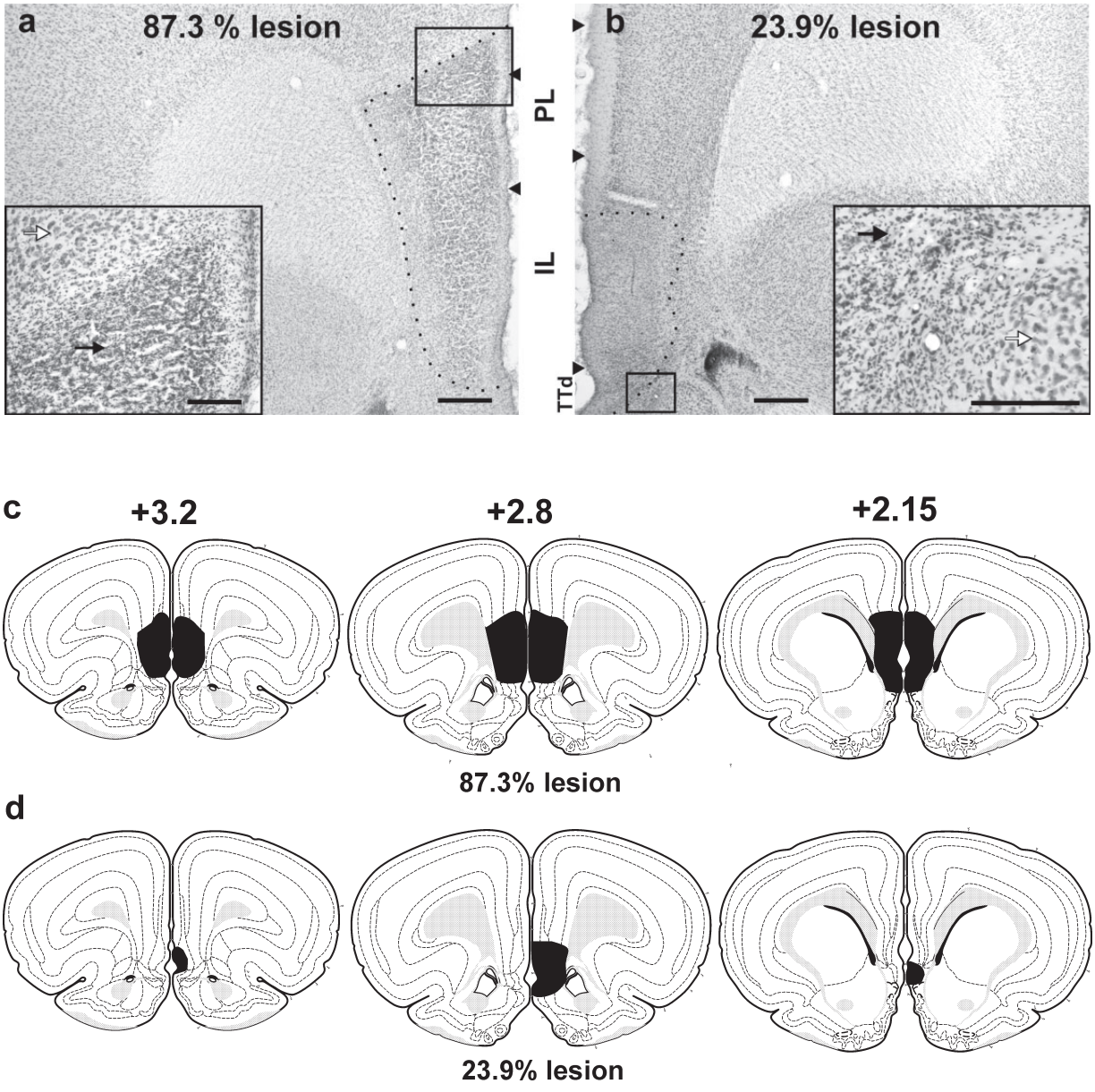

FIG. 3. The largest (87.3%, a and c) and smallest (23.9%, b and d) infralimbic area (IL) lesions are shown (see also Table 2 for the quantification of the lesions in different medial prefrontal areas for every rat). (a and b) Photomicrographs of Nissl-stained sections from the largest and smallest IL lesions (outlined by dotted lines). Arrowheads delimit the IL, prelimbic area (PL) and dorsal taenia tecta (TTd). The insets show at higher power the boundaries between healthy (white arrows) and damaged (black arrows) tissue. (c and d) Lesion extent in diagrammatic coronal sections taken from three levels of Swanson's atlas. Scale bars: a and b, 250 μm; bars in insets, 100 μm.

TABLE 2. Lesion size in medial prefrontal cortical areas, expressed as a percentage of the total surface of each cortical area

	Lesion size in medial prefrontal cortical areas (%)				
Rats	ACAd	PL	IL	ORBm	TTd
1	19.20	42.94	82.84	25.00	4.82
2	1.81	21.75	87.31	0.00	10.09
3	0.00	0.00	23.88	0.00	12.72
4	0.00	0.00	44.78	0.00	12.28
5	1.09	13.56	50.75	0.00	4.39
6	0.00	20.06	83.58	0.00	8.77
7	0.00	0.28	23.88	0.00	7.46
8	0.72	12.99	52.24	0.00	5.70
Bilateral surface area (mm^2)	2.76	3.54	1.34	1.44	2.28

ACAd, anterior cingulated area, dorsal part; PL, prelimbic area; IL, infralimbic area; ORBm, orbital area, medial part; TTd, taenia tecta, dorsal part. Bilateral surface area (mm^2) is shown in the bottom row.

The IL bilateral lesion did not change food intake under *ad libitum* feeding, as reported previously (Kolb & Nonneman, 1975; Recabarren *et al.*, 2005). Intact rats ate 18.87 ± 0.52 g/day whereas IL rats ate 19.18 ± 0.33 g/day. The circadian rhythms of locomotor activity and core temperature, as well as the 24-h accumulated locomotor activity, were not affected by the IL lesions (not shown), as previously reported by us for a different group of IL-lesioned rats (Recabarren *et al.*, 2005). However, IL lesions decreased the behavioral arousal and temperature responses induced by food enticing.

Intact rats (Valdés *et al.*, 2005) and rats with lesions smaller than 50% of the bilateral IL area showed a significant increase in locomotor activity, reaching more than twice the baseline level during the hour of food enticing (Fig. 4a). In contrast, those rats with lesions larger than 50% of the IL failed to increase their locomotor activity. Furthermore, we found that the increase in locomotor activity, which we used to assess behavioral arousal, was inversely related to lesion size (Fig. 4b).

Infralimbic cortex lesions also decreased the temperature responses to food enticing, an effect that was also dependent on the size of the IL lesion, as shown by the graphs in Fig. 5. We found that only the lesions larger than 50% of the IL abolished the thermal responses to food presentation to hungry rats, whereas the rats with smaller lesions increased their temperature (relative to the temperature at time 0) by 1.15 ± 0.43 °C, a value not different (*t*-test, $P = 0.2$) from the increase (0.6 ± 0.2 °C) reported for intact rats (Valdés *et al.*, 2005). A significant inverse correlation between lesion size and change in mean temperature relative to baseline (mean temperature during the first hour of presentation at the same local time but on the previous day) during food enticing is shown in Fig. 5c.

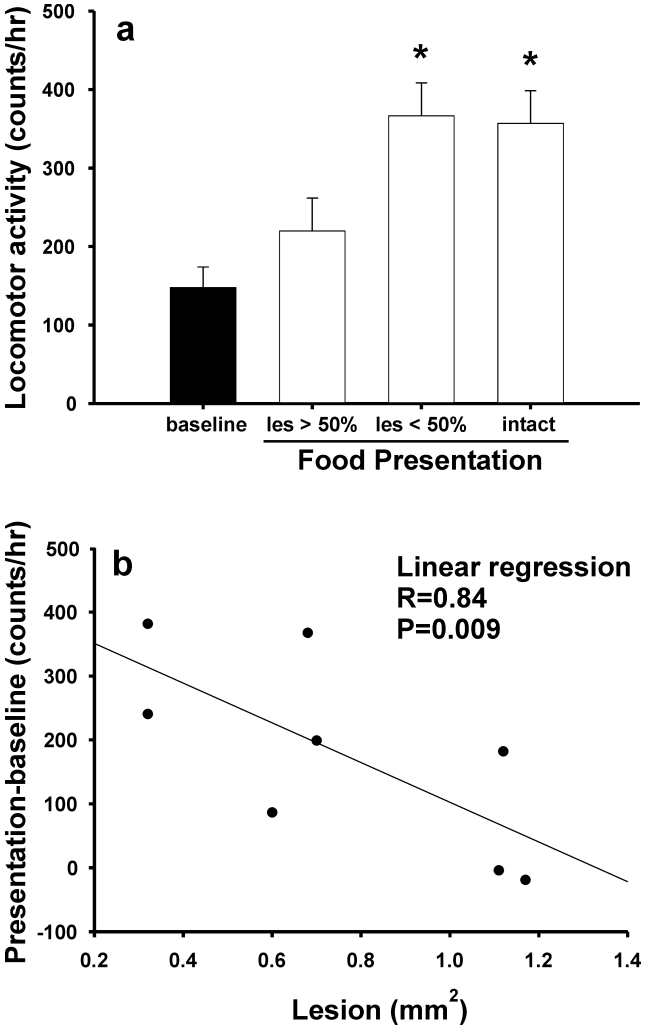

FIG. 4. Effect of infralimbic area (IL) lesion size (les) on locomotor responses to food enticing in hungry rats. (a) The accumulated locomotor activity during the first hour of food enticing of three groups of hungry rats. The baseline corresponds to the combined locomotor activity of the three groups the day before food presentation, during the same time of day as during the first hour of food presentation. They were pooled together because there were no significant differences between them. Note the significant increase in locomotor activity in the intact and small lesion groups relative to baseline (*$P < 0.05$) and the failure of the rats with larger IL lesions to increase locomotor activity. (b) Linear regression analysis revealed a significant inverse relationship between IL lesion size and behavioral response (total locomotor activity during the first hour of food presentation minus baseline, as defined above) to food presentation.

Effects of infralimbic area lesions on Fos immunoreactivity in the ascending arousal system

The group of 11 intact rats enticed for 2 h with food after a 24-h fast showed increased Fos IR in every nucleus or region of the ascending arousal system we assessed (Fig. 6a and b). These rats were used in a previous study (Valdés *et al.*, 2005) and the results are shown again here to contrast them with the effect of IL lesions on the same parameter. Animals with larger IL damage ($n = 4$) showed no increase in Fos IR after 2 h of enticing, whereas the subjects with smaller lesions showed small but significant increases in the number of Fos-immunoreactive neurons only in the tuberomammillary nucleus and the ventral tegmental area. The lower panels of Fig. 6 illustrate the effect of food enticing on Fos IR in selected ascending arousal system regions from an intact rat and a rat with a large IL lesion. We found no correlation between locomotor activity and amount of Fos IR in the ascending arousal system or between temperature responses and number of Fos-immunoreactive neurons in the subcortical regions involved in thermoregulation, probably because the drop in Fos IR was larger than the decrease in locomotor activity or temperature.

Effects of infralimbic area lesions on Fos immunoreactivity in subcortical regions controlling body temperature

We counted Fos-immunoreactive neurons in the preoptic area, dorsomedial hypothalamic nucleus, ventromedial hypothalamic

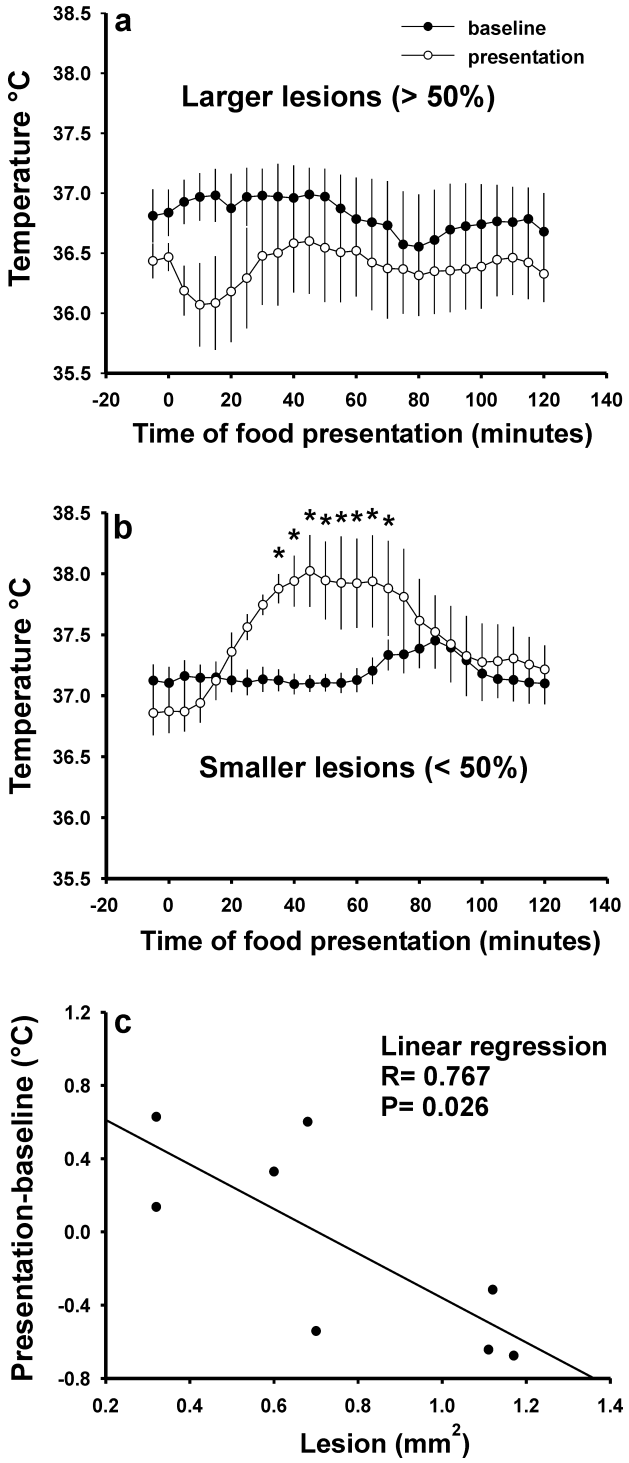

FIG. 5. Effect of infralimbic area (IL) lesion size on temperature responses to food enticing (from 0 to 120 min) in hungry rats. (a) Large IL lesions (> 50%; $n = 4$) prevented the increase in core temperature. (b) Food presentation at time 0 induced a significant (*) increase of nearly 1 °C in core temperature ($P < 0.05$ relative to baseline or to time −5 min) in rats with small IL lesions (< 50%; $n = 4$). (c) Linear regression analysis revealed a significant inverse relationship between IL lesion size and temperature response (mean temperature during the first hour of presentation minus mean baseline temperature) to food presentation.

nucleus and raphe pallidus, four brain regions that are concerned with thermoregulation and thermal responses that engage the sympathetic innervation to the brown adipose tissue. The brown adipose tissue is the effector of the thermal responses to food (Cannon & Nedergaard, 2004; Morrison, 2004). Intact rats showed increased Fos IR in the preoptic area, dorsal hypothalamic nucleus and raphe pallidus after 30 min of food enticing. Further increases in Fos IR after 60 and 120 min of enticing were observed, with minor variations in statistical significance (see Fig. 7). It is noteworthy that the time course of Fos IR in the ventromedial hypothalamic nucleus was similar to that of the thermal response, with both variables increasing only during the first 60 min of enticing. In contrast, in the other thermoregulatory nuclei the rise in Fos IR lasted 2 h, which is 1 h in excess of the hyperthermic response. The IL lesion abolished the increases in Fos IR in the four regions analysed.

Discussion

The present results indicate that the IL is essential in initiating the arousing and vegetative responses that underlie a feeding-related appetitive behavior and in activating their subcortical neural mechanisms. The IL was significantly active during this appetitive behavior and the bilateral lesion of the IL depressed the behavioral and thermal responses to enticing by food. The lesions had no effect on food intake, as reported previously (Kolb & Nonneman, 1975; Recabarren *et al.*, 2005), indicating that these rats had no problem with liking the food but rather failed to exert themselves trying to obtain it. The IL lesions also caused a concomitant suppression of Fos IR in subcortical structures that induce arousal and in subcortical regions involved in thermal responses. The failure of behavioral activation was accompanied, as expected, by a widespread depression of Fos IR in cortical and subcortical structures that were active in intact rats.

The ibotenic acid injections are known to kill local neurons by overexcitation while sparing axons of passage and glial cells (Jarrard, 1989; Fowler & Sherk, 2001). Our ibotenic acid injections predominantly damaged the IL, with destruction involving 23.9–87.3% of the IL, whereas the dorsally located prelimbic cortex and the ventrally located dorsal taenia tecta were largely spared. It is reasonable then to think that the effects of medial prefrontal cortex lesions reported here were due to the damage to the IL neurons and not to the damage of axons passing through the IL or the involvement of other cortical areas. Moreover, we found that the larger the IL lesion, the less behavioral arousal and thermal responses to enticing by food, indicating a causal relationship between IL integrity and these responses.

Our results show that the IL as well as many other cortical and subcortical areas had increased Fos IR in response to food enticing, but only if the rats were motivated for food by fasting. In stark contrast, both the satiated rats and the rats with IL lesion had little Fos IR in the brain, in parallel with the lack of behavioral activation. The fact that 36.6% of IL neurons increased their discharge rate during food enticing (Table 1) suggests that the high Fos IR observed in the IL under the same condition was related to food enticing. However, because we did not record single unit responses to food from the IL in satiated rats we cannot make an overall parallel between Fos IR and single unit responses. In addition, only 10% of the neurons responded to the empty box, which may reflect the lower Fos IR induced by the presentation of the empty box compared with the higher Fos IR after enticing with food. The recordings show that nearly one-third of IL cortical neurons were inhibited during one or more of the feeding conditions tested, a finding that cannot be reflected by Fos IR. It seems more likely that the high arousal of the hungry rats when trying to get the boxed food is a consequence rather than the cause of the widespread brain activation shown by a high Fos IR.

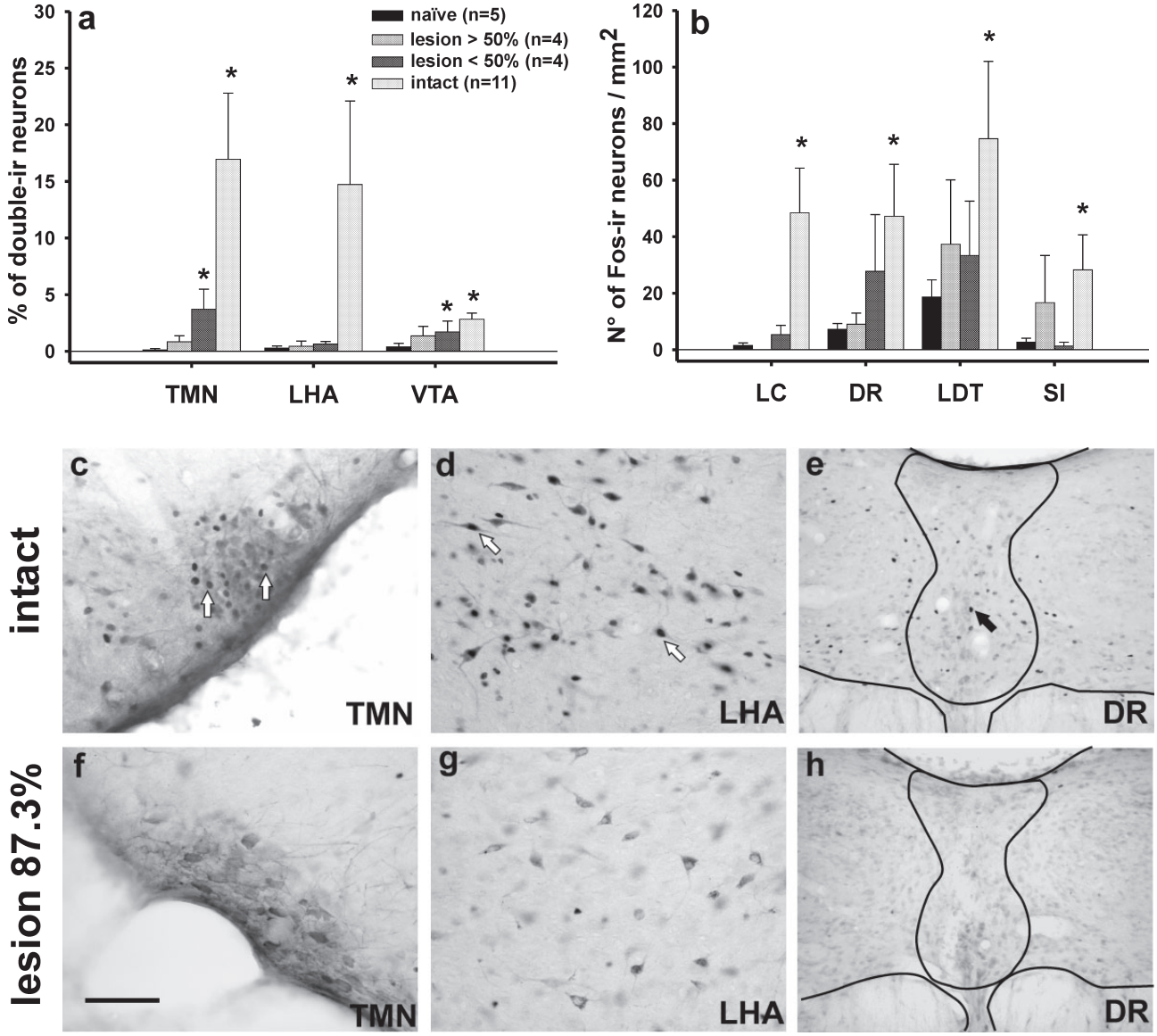

FIG. 6. Effects of infralimbic area (IL) lesion on the Fos immunoreactivity (IR) in the ascending arousal system nuclei of different groups of rats. All rats were presented with food for 120 min. Histograms depicting the percentage (a) or number (b) of neurons expressing Fos IR. Naive rats were used as controls for basal Fos IR. Food enticing significantly increased Fos IR in the intact group compared with naive controls. Large IL lesion completely abolished the rise in Fos IR, whereas the smaller lesions caused a large decrease in Fos IR. *Significant difference from naive rats ($P < 0.05$). Photomicrographs showing Fos IR in three AAS nuclei [tuberomammillary nucleus (TMN), lateral hypothalamic area (LHA) and dorsal raphe (DR)] in an intact rat (c–e), and in a rat with an 87.3% IL lesion (f–h). The TMN sections were also immunostained for adenosine deaminase (ADA) and the LHA sections were immunostained for orexin. White arrows point to neurons doubly labeled for Fos IR and ADA (c) or for Fos IR and orexin (d). Black arrow points to a Fos-immunoreactive neuron in the dorsal raphe (e). Note that the IL lesion abolished Fos IR in the three AAS nuclei. Bar, 50 μm. LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; SI, substantia innominata; VTA, ventral tegmental area.

The present results support the idea that the cortical feedback to the arousal system envisaged by Hebb (1955) on theoretical grounds and by GoldmanRakic (1987) originates from the prefrontal cortex, specifically from the IL. In our experimental paradigm to induce the appetitive phase in the hungry and intact rats, they showed increased alertness when food was presented, i.e. when it became a reachable goal, suggesting an appraisal of the situation that led them to try, sometimes frantically, to obtain the food pellets from the wire box. This appraisal and the subsequent plan for action are thought to be the result of prefrontal cortex activity and, as the present results indicate, the implementation of appropriate arousing and vegetative responses should require direct involvement of the IL, which receives processed information from other prefrontal cortices (Conde *et al.*, 1995; Gabbott *et al.*, 2003). The IL sends scant projections to other cortical regions so that its influence on cortical activation during appetitive behavior, unveiled by the lesions, should be explained by IL connections to brain regions that do provide extensive excitatory inputs, such as the nuclei of the ascending arousal system (AAS) (Jones, 2003).

A good candidate to promote arousal in response to IL signals in the present experimental situation is the tuberomammillary nucleus (Haas & Panula, 2003). We have shown (Valdés *et al.*, 2005) that the tuberomammillary nucleus is the first nucleus of the ascending arousal system that increases Fos IR while the appetitive behavior is occurring. These results supported the suggestions that histaminergic

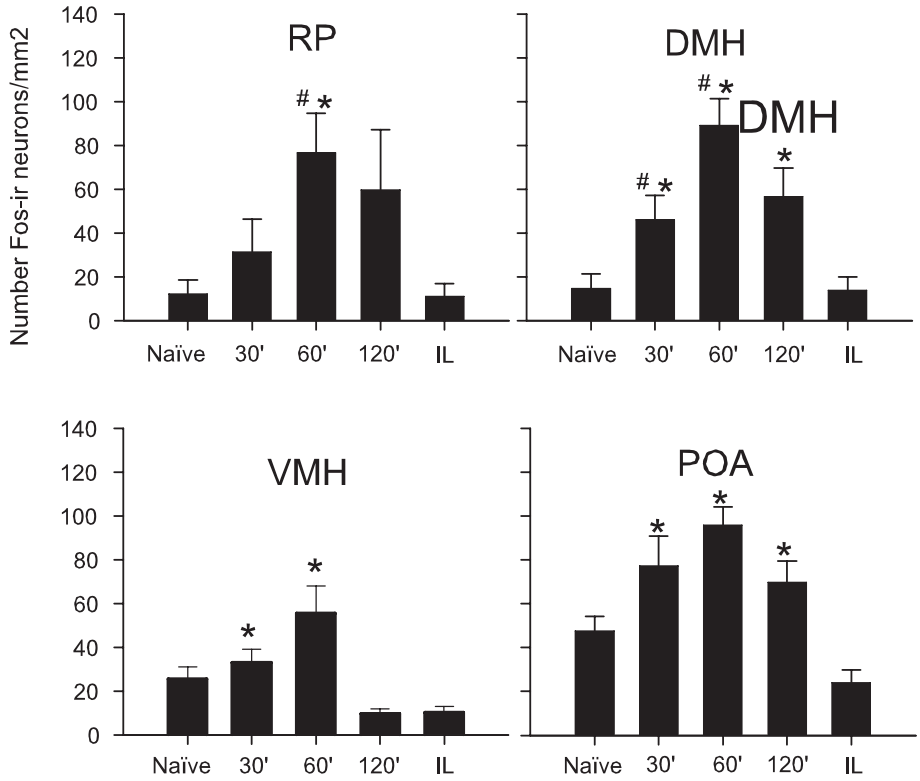

FIG. 7. Infralimbic area (IL) lesions suppressed Fos immunoreactivity (IR) in thermoregulatory nuclei. Intact rats were presented with food for 30, 60 or 120 min. IL lesion rats were enticed with food for 120 min. Naive rats were used as controls for basal Fos IR. Note that food enticing significantly increased Fos IR compared with naive controls and that IL lesion abolished the rise in Fos IR induced by enticing. *Significant differences with IL lesion; #significant differences with naive controls. DMH, dorsomedial hypothalamic nucleus; POA, preoptic area; RP, raphe pallidus; VMH, ventromedial hypothalamic nucleus.

neurons play a key role in exploratory behavior (Inoue *et al.*, 1996) and in maintaining vigilance faced with behavioral challenges (Parmentier *et al.*, 2002). In contrast, the tuberomammillary nucleus shows no Fos IR when satiated rats are enticed by food, when the animals are kept passively awake by sensory stimulation (Valdés *et al.*, 2005) or when they are subjected to restraint stress, LiCl-induced stress or amphetamine injections (Inzunza *et al.*, 2000). We hypothesized (Valdés *et al.*, 2005) that the tuberomammillary nucleus is activated by signals from the IL (Wouterlood *et al.*, 1987) that reflect an internal decision to engage in a motivated behavior, and that the other ascending arousal system nuclei are recruited later on if the appetitive behavior and arousal are prolonged. Behavioral arousal and anticipatory autonomic responses are a key component of the appetitive phase of motivated behaviors like feeding. In fact, it is difficult to imagine an animal that is driven to obtain a reward that seems attainable, or close at hand, which is not showing increased vigilance, propensity to move or responsiveness to sensory or emotional stimuli (Pfaff *et al.*, 2002).

In apparent contrast to the present results, we have shown in a previous work (Recabarren *et al.*, 2005) that IL lesion speeded up by a few days the onset of the food anticipatory locomotor activity that is normally observed under a restricted feeding schedule. These and previous (Mistlberger, 1994) results indicate that food anticipatory locomotor activity, which is under the control of circadian signals of unknown source, is expressed in rats with cortical lesions. Perhaps the explanation for the contrasting effects of IL lesion lies in the way by which the tuberomammillary nucleus gets activated. In the restricted feeding protocol the tuberomammillary nucleus is probably engaged by circadian signals from a food-entrained oscillator (Fig. 8) and the ensuing thalamo-cortical activation and arousal are the result of tuberomammillary nucleus activation. In agreement with the early onset of anticipatory activity under restricted feeding, the rats with IL lesion show premature responses and impulsivity (Risterucci *et al.*, 2003). In the present experiments the appetitive phase is induced by the presence of food (sensory signals) which involves appraisal of the rewarding value of these sensory signals by the orbitofrontal cortex (Schoenbaum *et al.*, 2003; Schoenbaum & Roesch, 2005), the decision to engage in food searching and the activation of the IL to increase arousal via the tuberomammillary nucleus and to produce appropriate autonomic responses, like the increase in thermogenesis (Fig. 8). Taken together, our lesion studies show that IL integrity is essential for the expression of increased locomotor activity in response to sensory cues (sight or smell of food) but that this cortex does not play such a role when circadian signals are involved in the initiation of food-seeking behavior.

The prefrontal cortex provides the cortical input to all the components of the ascending arousal system (Hurley *et al.*, 1991; Groenewegen & Uylings, 2000). It is interesting that whereas the IL seems to be the main source of cortical input to the tuberomammillary nucleus (Wouterlood *et al.*, 1987; Ericson *et al.*, 1991), many medial prefrontal areas provide cortical inputs to other ascending arousal system nuclei. The dorsal raphe for instance (Peyron *et al.*, 1998) has cortical inputs from cingulate, agranular insular, orbital, prelimbic and infralimbic cortical areas, whereas the locus coeruleus neurons show potent excitatory responses to stimulation of all areas in the medial prefrontal cortex (Jodo *et al.*, 1998). However, few double-labeled neurons were found in the infralimbic/prelimbic area after dual axonal tracing injections into the dorsal raphe and locus coeruleus (Lee *et al.*,

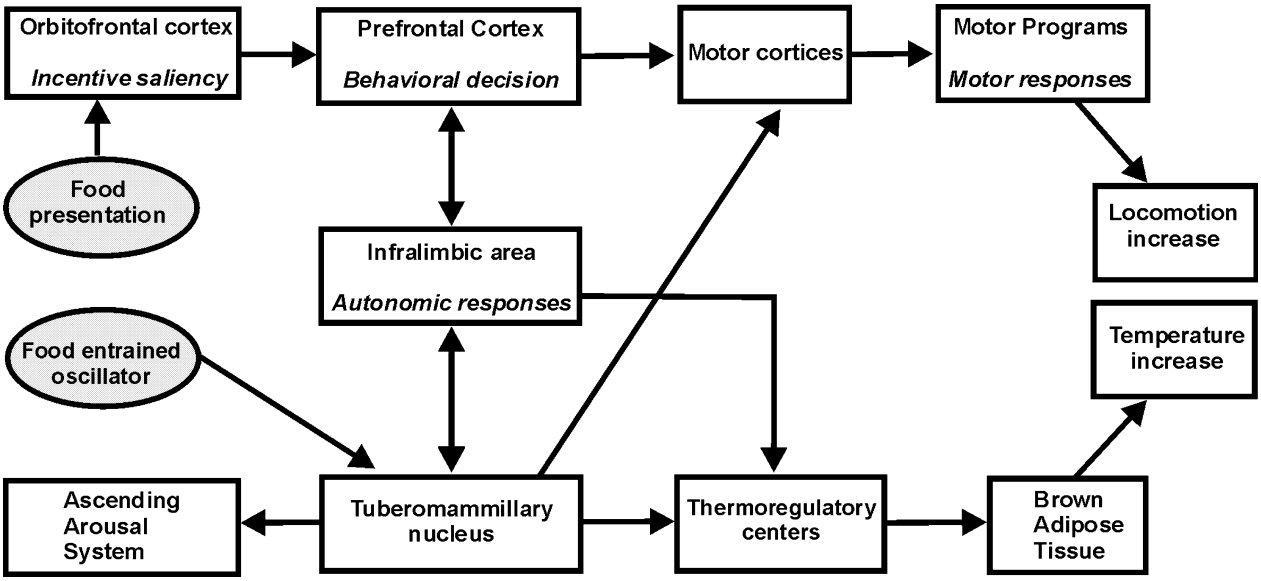

FIG. 8. A simplified and hypothetical wiring diagram indicating the initial targets for sensory or circadian stimuli signaling for food and the possible downstream pathways leading to locomotor and thermal responses to food signals. A key element is that the tuberomammillary nucleus is engaged by circadian signals when rats are entrained by scheduled feeding and by infralimbic area inputs during appetitive behavior.

2005), suggesting that neurons in these cortical areas send inputs to either nucleus.

The fact that the IL sends inputs to all AAS and not only to the tuberomammillary nucleus raises the question of why the tuberomammillary nucleus is the first ascending arousal system nucleus that becomes active during the appetitive behavior. A likely possibility is that different neuronal populations from the IL send efferents to specific nuclei of the ascending arousal system (Lee *et al.*, 2005) and that the IL neurons projecting to the tuberomammillary nucleus (Ericson *et al.*, 1991) may be especially responsive to feeding-related signals.

Our results confirmed (Recabarren *et al.*, 2005) that the IL is a key region mediating the rise in temperature that anticipates an upcoming meal and further show that the IL lesion eliminated the activation of hypothalamic and brainstem nuclei involved in temperature control. Taking these results together with work using cortical spreading depression (De Luca *et al.*, 1987; Monda *et al.*, 2004), the picture is emerging of prefrontal cortex at the top of the hierarchy of the brain structures controlling thermal responses. It is interesting that the time course of the thermal response to enticing with food was similar to that of Fos IR in the ventromedial hypothalamic nucleus but less so with that of the other thermoregulatory nuclei analysed. This observation concurs with work suggesting that the ventromedial hypothalamic nucleus is important in the thermogenic changes induced by cortical stimulation (Monda *et al.*, 2002) and feeding (Monda *et al.*, 1997).

The present study may contribute to a better understanding of the autonomic and/or arousal deficits present in a variety of human diseases affecting the prefrontal cortex, where dysfunction of Brodmann area 25 has been documented (Anderson *et al.*, 1999; Sullivan & Brake, 2003; Williams *et al.*, 2004). There is evidence in humans for impairments in autonomic responses after lesions to the ventromedial prefrontal cortex. Patients with such lesions, which included Brodmann area 25, thought to be homologous to the IL (Preuss, 1995; Ongur & Price, 2000; Uylings *et al.*, 2003), are unable to develop anticipatory skin conductance responses while evaluating gambling choices, a task requiring prefrontal cortex, but are able to generate skin conductance responses when they receive a reward or a punishment (Bechara *et al.*, 1999). These skin responses are viewed as a measure of general arousal (Venables & Christie, cited by Bechara *et al.*, 1999).

We are emphasizing here the role of the IL in arousal and appetitive functions. Nevertheless, the contribution of infralimbic/prelimbic areas to cognition and aversive responses has been amply studied. These medial prefrontal cortices contribute to behavioral flexibility and attention shifting (Ragozzino *et al.*, 1999, 2003; Delatour & Gisquet-Verrier, 2000), as is the case with tasks that involve changing attention between perceptual dimensions (e.g. between odours and texture) (Birrell & Brown, 2000) to obtain a reward. Indeed, the increased arousal mediated by the IL may contribute to the performance in those cognitive tasks, which include a motivation to obtain food rewards. The infralimbic/prelimbic cortical areas are also key structures in the control of aversive responses, like those elicited in stressful (McDougall *et al.*, 2004; Amat *et al.*, 2005) or fearful (Milad & Quirk, 2002) experimental paradigms. The present results extend the role of the IL to an appetitive behavior. In this context, a recent work on humans (Volkow *et al.*, 2005) has shown that Brodmann areas 11 and 25 have increased activity in cocaine-addicted subjects when craving and mood elevation were induced with methylphenidate.

One role for the medial prefrontal cortex is to anticipate coordinated adaptive autonomic changes to an upcoming task or behavior (Van Eden & Buijs, 2000). The rat IL has been considered an autonomic motor cortical area, based on connectional and functional studies (Hurley-Gius & Neafsey, 1986; Cechetto & Saper, 1990; Van Eden & Buijs, 2000). The present results on single unit recordings from the IL show that almost every neuron responded with increases or decreases in firing rate to one or more of the three experimental conditions, particularly to food presentation or after feeding. This finding is quite compatible with a role for the IL in controlling brain activity, through connections to the different ascending arousal system nuclei, and with a role in the control of bodily responses, which involve a variety of visceral and endocrine effectors that should be turned on or off to produce an appropriate overall response to the task at hand.

The present study demonstrates that the IL is a key cortical structure in appetitive behavior, which provides the signals to subcortical structures that trigger the preparatory responses in arousal and body temperature observed at the initiation of a feeding-related behavior.

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Abbreviations

AAS, ascending arousal system; IL, infralimbic area; IR, immunoreactivity; PBS, phosphate-buffered saline.

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