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# NUTRITIONAL STATUS MODULATES BEHAVIOURAL AND OLFACTORY BULB Fos RESPONSES TO ISOAMYL ACETATE OR FOOD ODOUR IN RATS: ROLES OF OREXINS AND LEPTIN

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**Abstract—Food odours are major determinants for food choice, and their detection depends on nutritional status. The effects of different odour stimuli on both behavioural responses (locomotor activity and sniffing) and Fos induction in olfactory bulbs (OB) were studied in satiated or 48-h fasted rats. We focused on two odour stimuli: isoamyl acetate (ISO), as a neutral stimulus either unknown or familiar, and food pellet odour, that were presented to quiet rats during the light phase of the day. We found significant effects of nutritional status and odour stimulus on both behavioural and OB responses. The locomotor activity induced by odour stimuli was always more marked in fasted than in satiated rats, and food odour induced increased sniffing activity only in fasted rats. Fos expression was quantified in periglomerular, mitral and granular OB cell layers. As a new odour, ISO induced a significant increase in Fos expression in all OB layers, similar in fasted and satiated rats. Significant OB responses to familiar odours were only observed in fasted rats. Among the numerous peptides shown to vary after 48 h of fasting, we focused on orexins (for which immunoreactive fibres are present in the OB) and leptin, as a peripheral hormone linked to adiposity, and tested their effects of food odour. The administration of orexin A in satiated animals partially mimicked fasting, since food odour increased OB Fos responses, but did not induce sniffing. The treatment of fasted animals with either an orexin receptors antagonist (ACT-078573) or leptin significantly decreased both locomotor activity, time spent sniffing food odour and OB Fos induction in all cell layers, thus mimicking a satiated status. We conclude that orexins and leptin are some of the factors that can modify behavioural and OB Fos responses to a familiar food odour.**


**Key words: olfaction, active wake, orexin receptors antagonist, leptin receptors, orexin A, immunohistochemistry.**

Most animals depend on olfaction when searching and choosing food, and the odours associated with food are essential to food intake control (Le Magnen, 1959). Various studies have demonstrated that the nutritional status of individuals influences odour detection: in primates and humans, fasting results in an increased perception of some food-related odours (Mulligan et al., 2002). In rodents, behavioural studies have demonstrated that olfactory sensitivity to a neutral odour increases in fasted rats (Aime et al., 2007). Indeed, odour signals are processed during multiple steps that start in the olfactory mucosa, where olfactory sensory neurons (OSNs) project to the first relay in the brain (mitral cells of the olfactory bulb) before being integrated in different areas of the CNS. The electrical activity of mitral cells varies as a function of nutritional status: fasting has been found to selectively increase mitral cell multiunit responses to food odour (Pager et al., 1972); in more recent studies, mitral cell single-unit responses were increased whatever the odorant (food or neutral) (Apelbaum and Chaput, 2003).

Numerous peripheral and hypothalamic peptides involved in food intake control vary according to nutritional status and may be responsible for modulating olfactory sensitivity. These include orexins, which are synthesised by neurons of the lateral hypothalamic area and are a stimulator of food intake (Sakurai et al., 1998; Edwards et al., 1999); the level of hypothalamic prepro-orexin mRNAs is maximal after 48 h of fasting (Cai et al., 1999). Orexin neurons project centrifugal fibres into the rat olfactory bulb (Nambu et al., 1999; Shibata et al., 2008). In narcoleptic human patients with decreased levels of orexin A (OxA), olfactory performance is impaired, and intranasal OxA treatment restores the olfactory function (Baier et al., 2008). When injected in the lateral cerebral ventricle of rats, OxA increases olfactory sensitivity (Julliard et al., 2007); orexin receptors are present in neurons of the bulb (Caillol et al., 2003; Hardy et al., 2005). Inversely, leptin is a potent satiety hormone, synthesised peripherally by adipocytes and acting on hypothalamic feeding networks to halt food intake (Shiraishi et al., 2000). In rats, i.c.v. leptin administration reduces food intake (Flynn et al., 1998) and olfactory sensitivity (Julliard et al., 2007); in mice, leptin modulates olfactory-mediated pre-ingestive behaviour (Getchell et al., 2006). We thus hypothesize that leptin may act on the olfactory bulb in a manner contrary to that of orexins, which may partly explain the modulation of olfactory performance by nutritional status.

The aim of the present study was thus to analyze possible modulations by nutritional status of both global behavioural responses and defined olfactory bulb cellular modifications induced by different olfactory stimuli. We chose isoamyl acetate as a neutral odorant capable of

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*Abbreviations:* BSA, bovine serum albumin; b.w., body weight; ICC, immunocytochemistry; i.c.v., intra-cerebro-ventricular; i.p., intra-peritoneal; IR, immunoreactive; OSN, olfactory sensory neuron; OxA, orexin A; PB, phosphate buffer; PBS, phosphate-buffered saline; PEG, polyethylene glycol; TX, Triton X-100.

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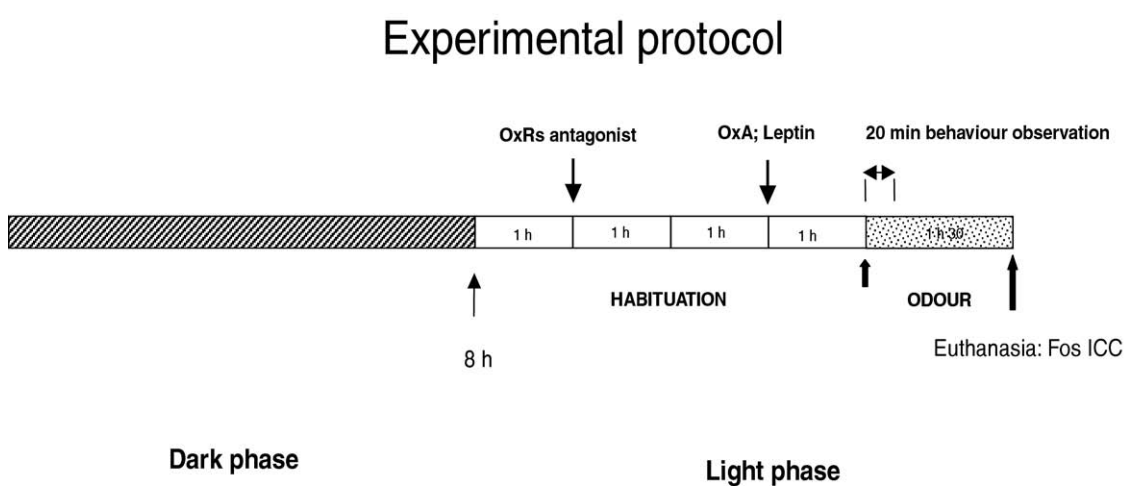


**Fig. 1.** Diagram representing the experimental procedures: after a 4-h habituation period (clean cage, no food pellets, under a fume hood), the odour stimulus was presented for 1 h 30 to 48-h fasted or satiated rats. Orexin receptor antagonist (ACT-078573) or vehicle was orally administered 3 h before the initiation of odour presentation; OxA, leptin or vehicle was administered i.c.v. 1 h before the initiation of odour presentation. Behavioural observations were performed during the first 20 min of odour presentation and rats were euthanized 90 min later.

stimulating large populations of sensory neurons in the mucosa, and food odour as a familiar biologically relevant stimulus. In terms of behavioural responses, we recorded locomotor activity and sniffing time. To measure the activity of cells in the different layers of the olfactory bulb, we chose immunohistochemical detection of the nuclear Fos protein. This product of the immediate-early gene c-fos, viewed as a third messenger, is widely employed as a functional anatomical marker of activated neurons and its expression is induced in the OB by the presentation of an odour (Sallaz and Jourdan, 1993; Guthrie and Gall, 1995).

The first part of our study was designed to investigate the effect of nutritional status (satiated vs. 48-h fasted rats) on both behavioural and OB Fos responses after presentation of an odour. The possible roles of orexins and leptin were then studied.

## EXPERIMENTAL PROCEDURES

### Animals

The experiments were performed on adult male Wistar rats, weighing 300–350 g. The rats were housed individually at a constant temperature (22 °C) with free access to food and water, under a 12-h light/dark cycle. A total of 60 rats were included in these experiments. All animals were handled in compliance with the principles of laboratory animal care and French laws on the protection of animals (law 87-848, decret du 13 février 2001). Every effort was made to minimize the suffering of rats and the number of animals.

### Drugs

OxA and leptin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted according to the manufacturer's guidelines. The rats received 3 nmol OxA in 3 $\mu$l saline (NaCl 0.9%) (Apelbaum et al., 2005), 5 $\mu$g leptin in 4 $\mu$l saline (Clark et al., 2006), or saline alone (vehicle) via i.c.v. cannulas (see below). The orexin receptor antagonist ACT-078573 (generous gift from Actelion Pharmaceuticals Ltd., Allschwil, CH) was formulated in polyethylene glycol 400 (PEG; Merck, Hohenbrunn, Germany) (vehicle). Rats received either the vehicle (1.75 ml) or ACT-078573 (100 mg/kg b.w. in 1.75 ml of vehicle) via gastric cannulae. Angiotensin II was purchased from Sigma-Aldrich and diluted in a saline vehicle.

### Surgery

One week before OxA, leptin or saline infusion, the rats were anaesthetized (equitezine, a mixture of chloral hydrate and pentobarbital, 0.3 ml/100 g body weight i.p.) and cannulas (Plastics One, 22-gauge stainless steel guide, Phymep, Paris, France) were implanted in the left lateral cerebral ventricle (Bregma: A=−0.8; L=−1.6; H=4). The correct positioning of the cannula was verified by an intense drinking response to an i.c.v. administration of angiotensin II (100 ng in 3 $\mu$l).

### Experimental protocol (Fig. 1)

For all experiments, at the beginning of the light phase, the rats were placed in clean cages under a laboratory fume hood as the standard ambient odour for a 4-h habituation period, with water but no food. The experiments were performed during the light phase since the Fos response to odours during the night (active period for rats) was significantly higher than during the day (Amir et al., 1999) and able to mask the modulation by nutritional status. At the end of the 4-h habituation period, different odour stimuli were presented in tea-balls: (i) control ambient odour (empty tea ball) (ii) unknown pure isoamyl acetate (150 $\mu$l on a filter paper; Montag-Sallaz and Buonviso, 2002; ISO unknown; Sigma-Aldrich) (iii) familiar isoamyl acetate (familiarisation for 20 min per day on 6 consecutive days in a cage containing litter odorized with 150 $\mu$l pure isoamyl acetate; Montag-Sallaz and Buonviso, 2002; ISO familiar) (iv) food odour (pellets in the tea ball). During odour exposure, the behaviour of rats was recorded for 20 min (time spent moving and sniffing the tea ball). After 90 min of odour exposure (peak of Fos protein induction after a stimulus; Kovacs, 1998), the rats were deeply anaesthetized and euthanized (Fig. 1).

The first experiment was designed to compare behavioural responses and Fos expression in the olfactory bulb after exposure to the different odour conditions (control, ISO unknown, ISO familiar, food odour) in rats either fasted for 48 h or fed *ad libitum* (satiated) ($n$=3 rats per group). This 48-h fasting period resulted in an 11% body weight decrease (318±5 vs. 281±5 g).

The second experiment was designed to test the role of orexins and leptin regarding behaviour and Fos responses to food odour. Satiated animals implanted with cannulas in the lateral

ventricle received OxA or vehicle 1 h before food odour stimulation. Orexin signalling was then blocked in fasted rats by administration via gastric cannulas of an antagonist of both OxR1 and OxR2 receptors or of vehicle (PEG 400), 3 h before odour stimulation. This antagonist passes the blood–brain barrier (Brisbare-Roch et al., 2007). The role of leptin was tested after i.c.v. administration in fasted rats 1 h before odour stimulation ($n=3$ rats for each experimental condition).

## Immunocytochemistry (ICC)

For all ICC assays, the rats were deeply anaesthetized with sodium pentobarbital (75 mg/kg i.p.) and perfused transcardially with 100 ml 0.9% NaCl (containing 0.1% sodium nitrite and 500 IUs of heparin), followed by 1000 ml of 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (PB; pH 7.4). The olfactory bulbs were removed rapidly and stored overnight at 4 °C in cryoprotectant solution (15% sucrose in PB). Tissues were frozen at −45 °C using isopentane cooled with liquid nitrogen, and embedded in Tissue Tek (Bayer Diagnostics, Puteau, France). Coronal cryostat sections of olfactory bulbs (14 μm) were collected on ready-to-use microscope slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany). Non-specific staining was blocked by incubation for 1 h at room temperature with nonimmune serum appropriate to the secondary antibody at a dilution 1:10 in 0.01 M phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 2% bovine serum albumin (PBS/TX/BSA). The sections were then incubated for 72 h at 4 °C with primary antibody in PBS/TX/BSA. The following primary antibodies were used: a rabbit polyclonal antibody to Fos protein (Ab-5, 1:20,000, Calbiochem, San Diego, CA, USA); a goat polyclonal antibody to Fos protein (1:100, SC52G, Santa-Cruz, Tebu, France); a rabbit polyclonal antibody to the long isoform of the leptin receptor Ob-Rb (1:80, Linco Research, St. Charles, MO, USA); a goat polyclonal antibody recognizing both long and short forms of the leptin receptor Ob-Rs (M18, 1:100, Santa-Cruz); a rabbit polyclonal antibody to GFAP (1:1000, DAKO, Trappes, France); rabbit polyclonal antibodies to orexin receptors (anti-OXR1, 1:1000, Alpha Diagnostic International, Cortec, France; anti-OXR1, 1:100, AB3092, Chemicon, Saint-Quentin-en-Yvelines, France for double-labelling; anti-OXR2, 1:200, Alpha Diagnostic International). Controls included incubation with nonimmune serum in place of each primary antibody. After washes in PBS, labelling was visualised using secondary antibodies conjugated with biotin (1:200, 90 min at room temperature) revealed either by an avidin–peroxidase kit (ABC standard kit; Vector, Burlingame, CA, USA) with diaminobenzidine (Sigma-Aldrich) (DAB 0.05%) and $H_2O_2$ (0.0015%), or by a streptavidin-conjugated fluorochrome. For double-labelling immunofluorescence, the two primary antibodies (goat anti-Fos protein and rabbit anti Ob-Rb receptor or rabbit anti-OXR1 receptor) were incubated together. The secondary antibodies (anti-goat FITC and biotinylated anti-rabbit raised in donkey) (Jackson ImmunoResearch, Interchim, Montluçon, France) were incubated together before the addition of Cy3-streptavidin (Sigma, 1:250). For DAB detection, the sections were air-dried, dehydrated in ethanol, cleared in xylene and mounted in DePex (BDH Laboratory Supplies, Poole, UK). For immunofluorescence detection, the preparations were washed in PBS and mounted in Vectashield-DAPI (Vector). Images were acquired on either a DMR Leica microscope equipped with an Olympus DP-50 CCD camera using Cell^F software (Olympus Soft Imaging Solutions GmbH, OSIS, Münster, Germany) or a Zeiss LSM 310 confocal microscope at 488 and 633 nm using helium–neon and argon ion lasers. Images were processed using Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA). Fos ICC images were acquired under standardized illumination and were not adjusted. Immunofluorescence images were adjusted for contrast and brightness to equilibrate light levels. The same settings were used for the zero primary control as for controls with primary antibody. In no case was the image content affected. Confocal observations were made at the Mima2 facilities in Jouy-en-Josas (France).

## Analysis of immunostaining

ImageJ software (rsbweb.nih.gov/ij) was used for Fos quantifications. In one olfactory bulb from each rat, images of six sampled sections were analysed in the rostrocaudal portion between 1.5 and 3.5 mm from the anterior pole. In each layer (glomerular, mitral and granular), the immunoreactive (IR) nuclei were plotted and their number was calculated using the method developed by Illig and Haberly (2003), with slight modifications (Fig. 2). Briefly, to compensate for differences in staining density, the background staining was subtracted for each image. A Fos-IR positive nucleus was defined using a 50% threshold between the darkest black nuclei (around 70) and the white level (255) (Fig. 2). The total number of IR nuclei in the six sections was calculated in each layer. The area of each layer was measured and the number of IR nuclei was converted into a cellular density.

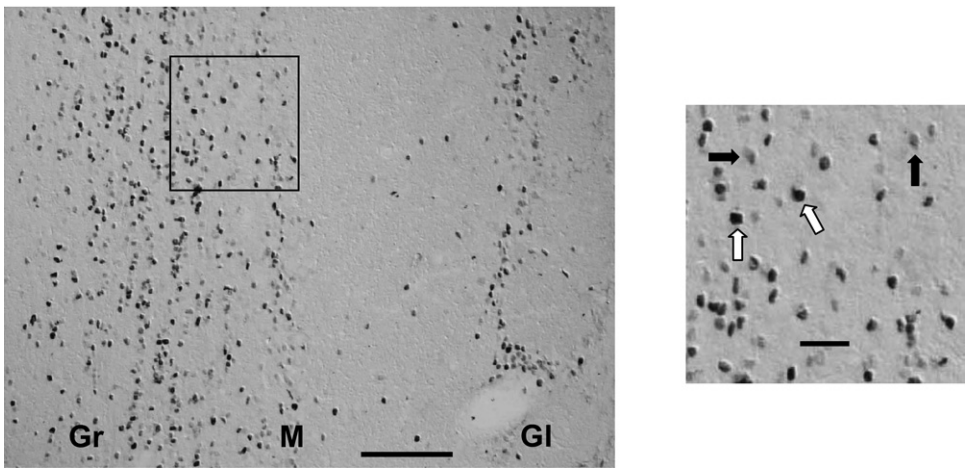


**Fig. 2.** Photomicrograph showing Fos nuclear labelling in the glomerular (Gl), mitral (M), and granular (gr.) layers of the olfactory bulb of a fasted rat submitted to unknown isoamyl acetate odour. Right panel: magnification of the selected zone in the left panel, exhibiting both positive (white arrows) and negative nuclei (black arrows). Scale bar=100 μm, left panel; 25 μm, right panel.

### Statistical analysis

For each experiment, in each layer of the bulb, cellular density values were normalized by considering the mean density in control rats as 100. Control rats were 48-h fasted ones, except for OxA experiments where the control rats were satiated ones. Behavioural and Fos data were analyzed using two-way ANOVA followed by the Holm-Sidak post hoc test or by Student's *t*-test when appropriate (Sigma-STAT software, Paris, France). *P*-values $<0.05$ were considered as significant.

## RESULTS

### Effect of nutritional status on behavioural and Fos responses of the olfactory bulb to a neutral or food odour

During the light phase, rats were mainly asleep. After 4 h of habituation in their cage in the experimental room, control fasted and satiated rats remained very calm during the 20 min of observation with the empty tea ball (ct) (Fig. 3A, locomotor activity). The presentation of odour stimuli (ISO unknown and familiar, food odour) elicited a spatial exploration in both groups. The time spent exploring the cage was significantly lower in satiated rats (* $P<0.05$); in the presence of food odour, this time was similar to that of control rats. When submitted to ISO stimuli, fasted and satiated rats sniffed the tea ball for a few seconds and remained in the other side of their cage (not shown). In contrast, fasted rats spent 50% of the time exploring the food odour whereas satiated rats remained quiet ($P<0.05$, Fig. 3B).

In Fig. 4 are presented both qualitative (A) and quantitative (B) Fos responses to odour stimuli. The olfactory bulbs of control fasted or satiated rats exhibited low levels of Fos expression (Fig. 4Aa, B). ISO unknown, ISO familiar and food odour resulted in Fos expression in periglomerular, mitral and granular cells layers. In all layers of the bulb, the maximal Fos response was due to ISO unknown stimulus (Fig. 4Ab, B); this response was similar in fasted and satiated rats (Fig. 4B).

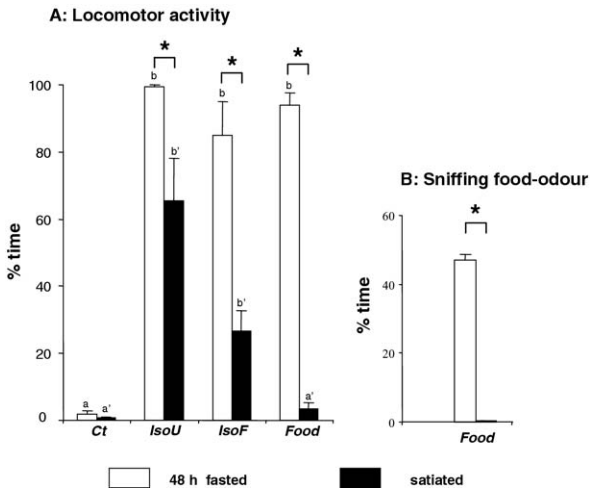


**Fig. 3.** Behavioural effects of two different odour stimuli (IsoU, IsoF: isoamyl acetate unknown or familiar; food odour; ct: control ambient odour, empty tea ball) in 48 h fasted (white bars) and satiated rats (black bars). Left panel: locomotor activity measured during 20 min following odour presentation. Right panel: % of time spent sniffing the tea ball containing the food odour stimulus. Values are mean±SEM for $n=3$ rats in each condition, and are compared using two-way ANOVA. Values with different superscripts differ significantly (odour effect: a≠b for fasted rats; a′≠b′ for satiated rats). * Same odour stimulus, fasted rats≠satiated rats.

The Fos expression was weaker with the familiar ISO and food odours (Fig. 4Ac, d, B). For these familiar stimuli, the density of Fos-ir neurons was significantly lower in satiated rats when compared with fasted rats, in mitral and granular cell layers (Fig. 4B). Interestingly, only a few glomeruli responded to the ISO stimulus; by contrast, the response to food odour seemed to be localised in many glomeruli, but the cells were only faintly labelled.

For the next experiments, we investigated the potential modulating roles of orexins and leptin regarding behavioural and FOS responses to food odour, this being considered as the most physiological stimulus.

### Effect of orexins and leptin on behavioural and olfactory bulb Fos responses to food odour

For these investigations, we attempted to mimic a fasted state in satiated rats by the injection of orexin, and to mimic a satiated state in fasted rats by treating them with either an OxRs antagonist or leptin.

Under control (empty tea ball) and food odour, satiated rats remained very quiet after a vehicle i.c.v. injection. When treated with i.c.v. OxA, the rats became very active and displayed spatial exploration of the experimental cage during 100% of time, whatever the odour stimulus (control or food) (Fig. 5A); they spent only 2.2%±1.3% of time exploring food odour whereas satiated control rats sniffed the tea ball for 0.8%±0.4% of time (Fig. 5A). The treatment of fasted rats using either an antagonist of both orexin receptors or leptin did not modify the behaviour of quiet fasted control rats with empty tea ball (Fig. 5B, C). After food odour presentation, rats treated with the vehicle explored the experimental cage and sniffed the tea ball; treatment with either the OxR1-OxR2 antagonist or leptin significantly diminished the duration of locomotor activity and the time taken to sniff the tea ball (Fig. 5B, C).

In Fig. 6 are presented qualitative (left panel) and quantitative (right panel) Fos responses to food odour in rats treated with OxA (A), OxRs antagonist (B) and leptin (C).

In vehicle-injected satiated rats, Fos immunoreactivity was very weak, even after the food odour stimulus (Fig. 6A, black bars of right panel). OxA per se greatly increased Fos expression in all layers of the olfactory bulb; however, the food odour stimulus resulted in a significant further increase in bulbar reactivity (Fig. 6A, dotted bars in right panel).

In control fasted rats (empty tea ball), the oral administration of an OxRs antagonist did not modify Fos expression in the olfactory bulb; the increased density of Fos neurons induced by food odour presentation in the three OB layers of vehicle-treated rats was significantly inhibited by OxRs antagonist.

The i.c.v. injection of leptin in control fasted rats induced a significant increase in the Fos response of mitral and granular cells (Fig. 6C, right panel). In rats treated with the vehicle, the presentation of food odour was followed by a significant

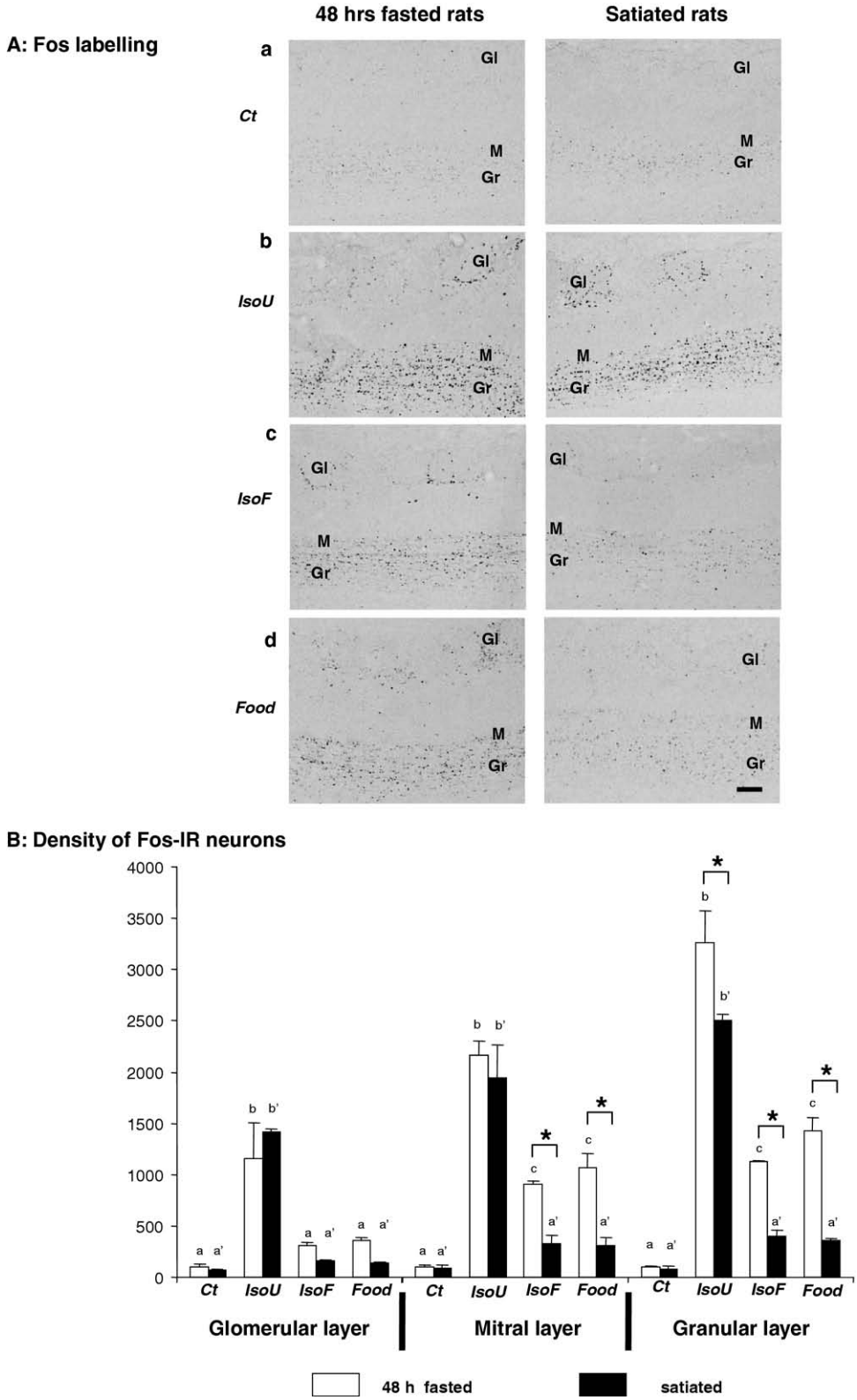


**Fig. 4.** (A) Photomicrographs illustrating Fos immunoreactivity in the olfactory bulb of 48 h fasted and satiated rats in response to (a) control ambient odour (Ct, empty tea ball); (b) unknown isoamyl acetate odour (IsoU) (c) familiar isoamyl acetate odour (IsoF); (d) food odour. Gl: glomerular cell layer; (M) mitral cell layer; Gr: granular cell layer. Scale bar=100 $\mu$m. (B) Quantitative analysis of Fos-ir neurons density in the Gl, M and Gr of olfactory bulbs from fasted (white bars) and satiated rats (black bars) exposed to different odour stimuli. Values are normalized to the values observed in each layer in fasted control rats. Values are mean±SEM for $n=3$ rats in each condition, and are compared using two-way ANOVA. Values with different superscripts differ significantly ($P<0.05$) (odour effect: a≠b≠c for fasted rats; a′≠b′ for satiated rats) * Same odour stimulus, fasted rats≠satiated rats.

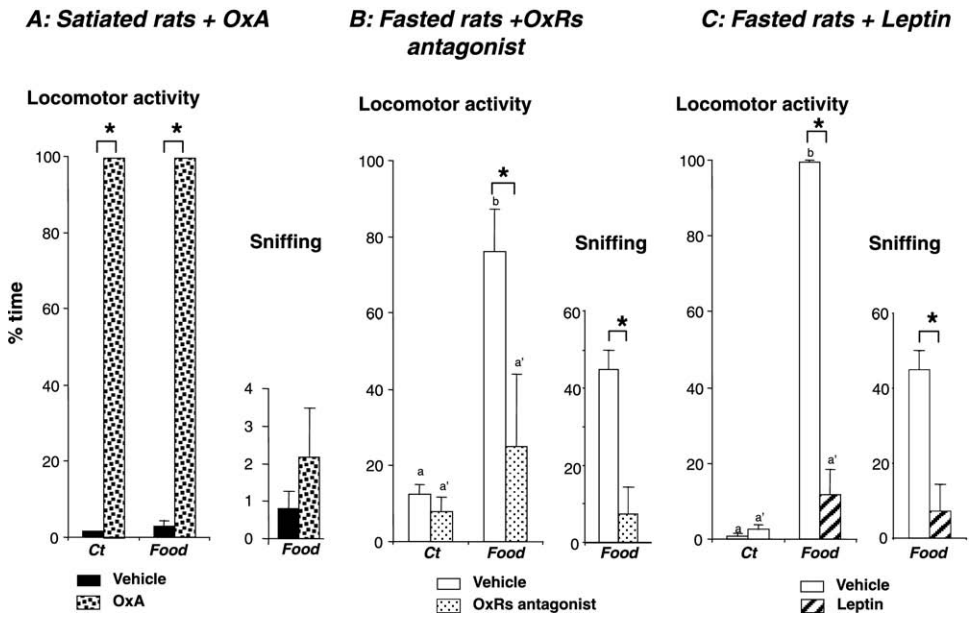


**Fig. 5.** Behavioural effects of food odour stimulus in satiated rats treated with OxA (A), and in fasted rats treated with OXRs antagonist (B) and leptin (C). Locomotor activity was measured during the 20 min following odour presentation; control (Ct, empty tea ball) or food odours were presented to vehicle and OxA-, OXRs antagonist–, and leptin-treated rats. The % of time spent sniffing the tea ball containing food pellets was measured for vehicle and treated rats (for OxA-treated group, please note the different scale). Values are mean±SEM for $n=3$ rats under each condition, and are compared using two-way ANOVA for locomotor behaviour and Student's *t*-test for sniffing behaviour. Values with different superscripts differ significantly (odour effect: a≠b for vehicle-treated rats). * Same odour stimulus, vehicle-treated rats≠OxA-, OxRs antagonist– or leptin-treated rats.

increase in FOS-positive cells in the three layers of the bulb. However, the response of the olfactory bulb to food odour was completely suppressed after treatment with leptin.

### Localisation of orexin and leptin receptors in the olfactory bulb (Fig. 7)

The implication of orexins and leptin in the modulation of olfactory bulb responses to food odour encouraged us to try to clarify the possible direct targets of these peptides. As already published, (Caillol et al., 2003; Hardy et al., 2005), OxR1 and OxR2 receptors were found to be strongly expressed in some mitral cells (Fig. 7A, C). For OxR1, a faint labelling was also present in many periglomerular and granular cells (Fig. 7A, B). Double labelling (OxR1-Fos) was performed on olfactory bulbs from fasted rats stimulated by food odour (Fig. 7D) and revealed Fos-positive nuclei in some mitral cells IR for OxR1. The numerous Fos-positive nuclei in the granular layer were not localised in OxR1-ir neurons.

When an antibody specific to the long form of leptin receptor (Ob-Rb) was used, faint labelling was observed in some mitral and granular cells (Fig. 7E). However, when an antibody recognizing both the long and short forms of leptin receptor was used, the labelled cells were astrocytes, as shown by co-localisation with GFAP (Fig. 7F, G, H). These astrocytes were mainly localised in glomerular structures, but were also found in the granular layer (Fig. 7F).

The double labelling (Ob-Rb-Fos) performed on olfactory bulbs from fasted rats stimulated by food odour revealed Fos-positive nuclei in a few mitral cells. Again, the numerous Fos-positive nuclei found in the granular layer were not localised in Ob-Rb IR cells (Fig. 7I).

## DISCUSSION

Taken together, our results demonstrate complex interactions between nutritional status of an animal and its behavioural and neuronal olfactory bulb responses to two different odour stimuli (ISO unknown or familiar, and food odour). We reveal a discrepancy between pure olfactory processing as tested by new ISO stimuli, resulting in increased locomotor reactions and a large increase in Fos responses in the bulb, whatever the nutritional status, and olfactory processing of a palatable stimulus (food odour) or of a familiar ISO odour, both linked to nutritional status.

Our behavioural observations confirmed ISO as a neutral odour, not eliciting sustained sniffing even in fasted rats, in contrast to food odour. Fos immunohistochemistry allowed us to demonstrate that neuronal reactivity in the layers of the bulb is regulated by nutritional status. Furthermore, we demonstrated for the first time the role of orexins and leptin in nutritional modulations of food odour responses, both at the behavioural and olfactory bulb neuronal levels.

### Odour stimuli, nutritional status, behavioural and olfactory bulb Fos responses

In this part of the work, we used ISO as an unknown or a familiar neutral odour. We found that this stimulus induced an increase in locomotor activity, probably due to the perception of the odour, in fasted and satiated rats, but no sniffing behaviour. The higher locomotor response observed in fasted rats could be linked to a higher neocortical arousal resulting from odour presentation upon fasting (Gervais and Pager, 1979, 1982).

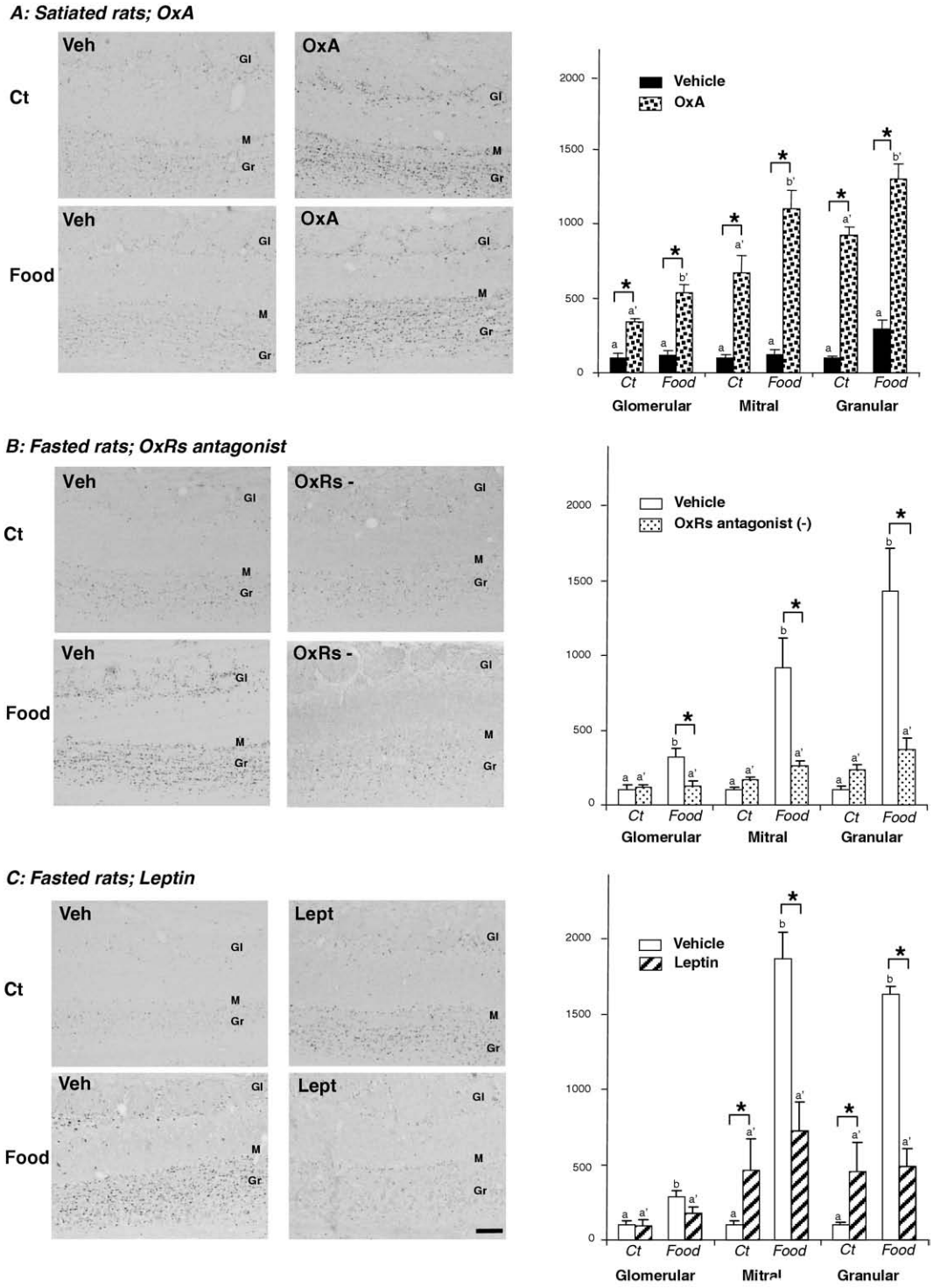


**Fig. 6.** Qualitative (left panels) and quantitative analysis (right panels) of Fos responses to food odour in satiated rats treated with i.c.v. OxA (A) and in fasted rats treated orally with OxRs antagonist (B) or i.c.v. with leptin (C). The effects of empty tea ball (Ct) and of food odour are reported for vehicle and treated rats ($n=3$ for each condition). For quantitative analysis, values are normalized to the values observed in each layer in satiated vehicle-treated rats (A) and in fasted vehicle-treated rats (B, C). Values are mean±SEM and are compared using two-way ANOVA. Values with different superscripts differ significantly (odour effect: a≠b for vehicle-treated rats; a′≠b′ for treated rats). * Same odour stimulus, vehicle-treated rats≠OxA-, OxRs antagonist– or leptin-treated rats. Gl: glomerular cell layer; M: mitral cell layer; Gr: granular cell layer. Scale bar=100 μm.

We also used food odour as a familiar, palatable and complex odour. This stimulus strongly increased both exploratory and sniffing behaviours, in fasted rats only. Indeed, in these rats, a food odour stimulus is very mean-

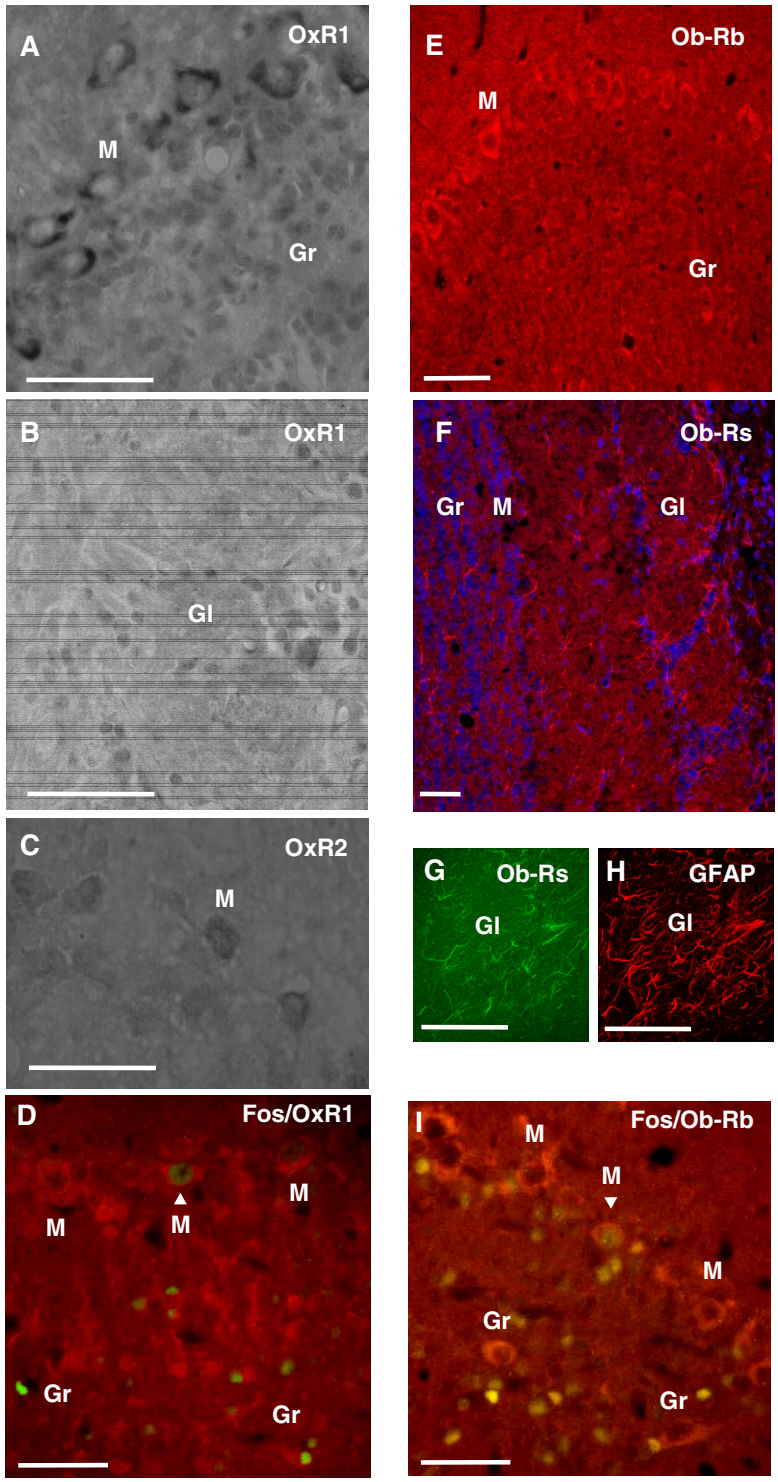


**Fig. 7.** Photomicrographs illustrating the cellular localisation of orexin receptors (left panel) and leptin receptors (right panel) in fasted rats. Immunoreactivity for OXR1 receptor was evidenced by DAB deposits in Ms and Grs (A), and in periglomerular cells (B). A faint immunoreactivity for OXR2 (DAB deposits) was present in some Ms (C). A double-labelling immunofluorescence experiment (D) showed Fos-ir nuclei (green) in the olfactory bulb of a fasted rat submitted to food odour: one M expressing OXR1 receptor (red, white arrowhead) was Fos-ir; other ones did not respond to food odour. In the Gr, numerous Fos-ir nuclei were present but not localised in OXR1-positive cells. Immunoreactivity for the long form of leptin receptor, Ob-Rb, was localized in numerous Ms and a few granular cells (red; E; Linco antibody). The localisation of short and long-forms of the receptor Ob-Rs was performed using the Santa-Cruz M18 antibody. Labelling was present in all layers of the olfactory bulb (F; red). Confocal images of a double-labelling experiment revealed that Ob-Rs positive cells (green, G) were astrocytes evidenced by GFAP (red, H). Double-labelling immunofluorescence (I) in olfactory bulb of fasted rats submitted to food odour showed a Fos-ir nucleus (green) localized in one Ob-Rb positive M (white arrowhead; red); numerous Fos-positive nuclei were observed in granular cells which did not express Ob-Rb. M: mitral cell; Gl: glomeruli; Gr: granular cell layer. Scale bars=50 $\mu$m.

ingful; this result further demonstrates that very meaningful stimuli, such as a predator odour in satiated rats (Cattarelli and Chanel, 1979) or food odour in fasted ones, results in a strong awaking influence (Gervais and Pager, 1979, 1982).

The discrepancy between locomotor and sniffing behavioural responses for ISO and food odours emphasizes the difference between the perception of the olfactory stimulus, able to induce an exploratory behaviour in quiet rats and to increase Fos expression in olfactory bulbs, and sniffing behaviour occurring only for a pleasant meaningful odour.

Our evaluation of Fos expression in the olfactory bulb provides new quantitative data on the cellular targets for modifications of olfactory coding in the brain by nutritional status. This methodology allowed us to observe the activation of all categories of OB neurons in response to odours in non-anaesthetized animals.

We showed a high response level after unknown isoamyl acetate stimulation in glomerular, mitral and granular cell layers of olfactory bulb, probably due to coincidence between pure olfactory response and activation of centrifugal inputs (Montag-Sallaz and Buonviso, 2002). This high response was significantly reduced after familiarisation and became equivalent to the response induced by food odour. This effect of familiarisation is in agreement with a decreased electrophysiological response of mitral/tufted cells (Buonviso and Chaput, 2000) and with a decreased c-fos mRNA expression in granular cell layer (Montag-Sallaz and Buonviso, 2002).

The use of Fos labelling also provided information on the pattern of glomeruli activated by a pure odorant or a complex food odour: only a few glomeruli responded to ISO stimulus; by contrast, the response to food odour seemed to be localised in numerous glomeruli, but with periglomerular cells faintly labelled. Using intrinsic signal imaging, Lin et al. (2006) clearly showed that natural odorant stimuli resulted in the activation of a complex panel of glomeruli.

Our results clearly demonstrated a modulating role of nutritional status on Fos olfactory bulb responses to familiar ISO and food odours. In satiated animals, stimulation by familiar ISO or food odour did not modify the density of Fos-ir neurons in all layers of the bulb, whereas it resulted in strong responses of both the mitral and granular cell layers in fasted rats. Our results in the mitral cell layer were in agreement with the results obtained by Pager et al. (1972); Gervais and Pager (1982); Apelbaum and Chaput (2003), all demonstrating an increased electrophysiological response by fasting. To our knowledge, the marked increase in granular cell responses to food odour in fasted animals has never previously been described. Our results are also in agreement with those of Aime et al. (2007), who demonstrated a higher detection of familiar aversive ISO stimulus in fasted animals than in satiated ones.

### Modulation by orexins and leptin

The olfactory bulb expresses numerous neuropeptides linked to the nutritional status as well as their receptors. We and others (Sakurai, 1999; Caillol et al., 2003) have localized orexin fibres and their two receptors in different neuronal populations of the bulb, and, during the present work, we demonstrated for the first time the presence of various leptin receptor isoforms in some mitral and granular cells, and in numerous astrocytes. We then decided to study the modulating role of these peptides on behavioural and olfactory bulb responses to the food odour.

The simplest way to study such a potential role for orexins is to inject the peptide i.c.v. to satiated rats. However, the intrinsic role of the peptide, both on behavioural and neuronal Fos responses, largely interferes with the demonstration of possible modulation of food odour processing by OxA. Indeed, in satiated OxA-treated rats, we observed a large increase in both locomotor activity and Fos induction in all layers of the olfactory bulb, even in the absence of food odour stimuli. This high level of OxA Fos responsive neurons is partly due to the presence of OxRs in the different OB cell layers (Caillol et al., 2003; Hardy et al., 2005). The high locomotor activity after OxA treatment is in agreement with data demonstrating that central orexin administration in rat results in an increase in locomotor activity (Nakamura et al., 2000) and wakefulness (Hagan et al., 1999). Furthermore, orexin neurons in the lateral hypothalamus area have projections to brain arousal areas (Akanmu and Honda, 2005; Harris and Aston-Jones, 2006; Adamantidis and De Lecea, 2008a,b). The presentation of food odour in OxA-treated rats resulted in a further increase in Fos responses; however, it did not induce sniffing behaviour. This prompted us to use an inverse approach, i.e. to block the orexin pathways in fasted rats by using an antagonist of both orexin receptors (ACT-078573, Brisbare-Roch et al., 2007). We found that after OxRs antagonist treatment, both the behavioural (locomotor and sniffing) and Fos responses of the bulb were similar to those observed in satiated rats after food odour exposure. This clearly demonstrates the implication of orexin system in modulating food odour processing. Our results are in agreement with behavioural data demonstrating an increase in olfactory detection performances of an aversive ISO odour in fasted rats, mimicked by an orexin injection in satiated ones (Aime et al., 2007; Julliard et al., 2007). They also agree with the electrophysiological studies demonstrating a direct effect of OxA on a discrete population of mitral cells, and an indirect effect on periglomerular-granular cells (Apelbaum et al., 2005; Hardy et al., 2005).

To our knowledge, there are no published data reporting a possible effect of leptin on the olfactory bulb. We showed here that food odour stimulation in leptin-treated fasted rats increased neither locomotor and sniffing activity nor Fos expression in olfactory bulb, mimicking what was observed in satiated rats. Getchell et al. (2006) and Julliard et al. (2007) have already demonstrated an inhibitory role of leptin in olfactory-driven food detection and sensitivity to aversive ISO odour.

The capacity of both orexins and leptin to modulate the olfactory bulb responses to food odour could be due to a variation either of the peptides levels or of the number of their receptors locally expressed. The quantification of the

transcripts coding for OxR1, OxR2, and two isoforms of the leptin receptor (Ob-Ra and Ob-Rb) by real-time quantitative PCR showed similar expression levels of these receptors in satiated and 48-h fasted rats (not shown). The nutritional modulation of olfactory bulb responses to food odour stimuli is thus due, at least in part, to local variations of orexins and leptin. However, after food odour presentation of fasted animals, double labelling experiments revealed few examples of Fos-ir neurons expressing OxR1 or Ob-Rb in the mitral cell layer. Thus, the Fos response occurred mainly in neurons neither OxR1-ir nor Ob-Rb-ir, suggesting that the modulating role of orexins and leptin could be of an indirect nature.

### Extra-bulbar targets of nutritional cues

A 48-h period of fasting results in important modifications of circulating hormones and metabolites, which have significant consequences on the first levels of olfactory processing, the olfactory mucosa (Baly et al., 2007; Lacroix et al., 2008) and bulb, and also on central regions of the brain sending centrifugal inputs to the bulb.

An odour stimulus first activates OSN in the mucosa. These neurons make synapses with mitral cells, which are in a close relationship with periglomerular cells. Therefore, Fos induction after odour stimulation in mitral and periglomerular cells is mainly due to OSN activation. The roles of orexins and leptin in modulating the olfactory message could thus be due partly to the action of these peptides at the very first level of signalling pathways, the OSN. We and others have localized orexin and leptin receptors on cilia of OSN (Caillol et al., 2003; Getchell et al., 2006; Baly et al., 2007). Electro-olfactogram studies have demonstrated that orexins increase and leptin decreases the depolarisation of olfactory mucosa in response to isoamyl acetate odour (Meunier et al., personal communication; Savigner et al., 2009).

The three different layers of the olfactory bulb receive direct afferent fibres from the rest of the brain: granular cell layer from locus coeruleus noradrenergic neurons (McLean et al., 1989; Bouna et al., 1994), glomerular cell layer from raphe serotoninergic neurons (McLean and Shipley, 1987), and all layers from lateral hypothalamus orexinergic neurons (Nambu et al., 1999). The olfactory bulb also receives indirect polysynaptic information from nucleus of the tractus solitarius (NTS) (Guevara-Aguilar et al., 1982, 1987) and an electrical stimulation of the vagus nerve modifies the electrical activity of periglomerular cells (Garcia-Diaz et al., 1984). Furthermore, the Fos induction by an odour stimulus in granular cell population is mainly due to afferents of central origin as demonstrated by olfactory peduncle sectioning (Sallaz and Jourdan, 1996). All these brain regions are potential targets for nutritional cues, via orexins and leptin variations, since they express both orexin (Trivedi et al., 1998; Bourgin et al., 2000; Greco and Shiromani, 2001; Marcus et al., 2001; Liu et al., 2002) and leptin receptors (Elias et al., 2000; Hay-Schmidt et al., 2001; Williams and Smith, 2006; Huo et al., 2008), suggesting numerous possible indirect sites for the orexin and leptin modulation of olfactory bulb responses.

Forty-eight hours of fasting may be necessary to enhance the effect of factors such as orexin and leptin, whose individual effect may be blurred during shorter periods. Noticeably, the decrease in plasma leptin and insulin, glucose and triglycerides, combined with enhanced local orexin production may be responsible for a coordinate enhancement of bulbar response in every layer controlling the transmission of the olfactory message, thus disclosing a concerted metabolic control that results in an adaptive tuning of the olfactory response to the animal physiological status.

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