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Chronic restricted access to food leading to undernutrition affects rat neuroendocrine status and olfactory-driven behaviors ☆

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ABSTRACT

Previous studies have demonstrated that olfactory-driven behaviors in rats are influenced by short-term caloric restriction, partly through the modulation of olfactory sensitivity by appetite-modulating hormones or peptides such as insulin and leptin. Here, we addressed the issue of a long-term modulation of their neuroendocrine status by evaluating the effect of chronic food restriction in rats following a limitation of the duration of daily food intake to 2 h (SF) instead of 8 h (LF) on the expression of insulin and leptin system in the olfactory mucosa and bulb and on olfactory behaviors. This restriction resulted in a one-third reduction in the daily food intake and a 25% reduction in the body weight of SF rats when compared to controls, and was accompanied by lower levels of triglycerides, glucose, insulin and leptin in SF rats. Under these conditions, we observed a modulation of olfactory-mediated behaviors regarding food odors. In addition, restriction had a differential effect on the expression of insulin receptors, but not that of leptin receptors, in the olfactory mucosa, whereas no transcriptional change was observed at the upper level of the olfactory bulb. Overall, these data demonstrated that long-term changes in nutritional status modulate olfactory-mediated behaviors. Modulation of insulin system expression in the olfactory mucosa of food restricted rats suggests that this hormone could be part of this process.

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Introduction

Most animals rely on olfactory cues to locate food sources, and to discriminate and determine the palatability and hedonic value of food. In humans, the hedonic value of food plays a critical role in the regulation of appetite and food intake, and changes in nutritional status can thus be expected to modify reactions to odors. Indeed, it is well known that the pleasantness of food and food-related cues is largely modulated by the nutritional and metabolic status of individuals, a phenomenon called alliesthesia (Cabanac and Fantino, 1977; Jiang et al., 2008): according to these authors, in a hunger state, the pleasantness of food odors is increased. However, although some studies have concluded that olfactory sensitivity to food-related odors is increased in fasted versus satiated states (Albrecht et al., 2009), other data have shown that olfactory sensitivity to neutral odors is increased in a hunger state or in individuals with a low body mass index (BMI), whereas for food odors, olfactory sensitivity is greater in a satiated state in individuals with a high BMI rather than those with a low BMI (Stafford and Welbeck, 2011). Short-term hyperinsulinemia mimicking the postprandial state raises the olfactory threshold, reducing smelling capacity and impairing the pleasantness of food (Ketterer et al., 2011). In addition, peripheral adipokines (leptin and adiponectin) and gut hormones (ghrelin) may alter the perception and pleasantness of specific odors, either directly via their receptors in the olfactory system, or through brain regions such as the piriform cortex, the orbito-frontal cortex which integrate sensory, metabolic, memory and motivation information (Schloegl et al., 2011; Trellakis et al., 2011).

In rodents, the modulation of olfactory-driven behaviors by the feeding state is now well established and the involvement in this phenomenon of metabolic molecules originating from the central nervous system or the periphery is largely documented. During the past ten years, numerous studies have provided a molecular basis for links between hormones of energy balance, functioning of the olfactory system and resulting olfactory-driven behaviors. Opposite effects of appetite-suppressing and appetite-stimulating peptides have been described on both olfactory sensitivity and olfactory-driven behaviors. Our group demonstrated the presence of receptors for

Abbreviations: SF, short feeding; LF, long feeding; OM, olfactory mucosa; OB, olfactory bulb; ICV, intracerebroventricular; BMI, body mass index.

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several metabolism-related molecules (such as orexins, leptin and insulin) at the two first levels of the rat olfactory system: the olfactory mucosa (OM) in the nasal cavity and the olfactory bulb (OB) in the forebrain (Baly et al., 2007; Caillol et al., 2003; Lacroix et al., 2008; Prud'homme et al., 2009). Their localizations on several types of cells, such as olfactory sensory neurons in OM or mitral cells in OB, strongly support the large participation of their ligands in the control of olfactory sensitivity. Interestingly, we showed that the level of expression of some of these receptors was modified or modulated by the nutritional status of the animals. In the OM, a prolonged fasting period of 48 h led to an increase in the levels of expression of both leptin and insulin receptors (Baly et al., 2007; Lacroix et al., 2008). In these fasted animals, we also observed that olfactory-driven behaviors are exacerbated; they were inhibited by the intracerebroventricular (ICV) injection of leptin or by the oral administration of orexin receptor antagonists mimicking a fed state (Prud'homme et al., 2009). Moreover, ICV administration of orexin in fed animals (mimicking a fasted state) increased food olfactory-driven behaviors. Therefore, an acute modification of the feeding status, through food deprivation or use of an anorexigenic or orexigenic treatment, affected the olfactory sensitivity of the rat olfactory system.

Some data have indicated that more subtle modifications to nutritional status may also be able to modulate olfactory sensitivity: rats fed for only 4 h a day and tested either 4 h before or 2 h after the meal displayed significant differences in olfactory sensitivity (Aime et al., 2007), this sensitivity being increased in the fasted rats. These modifications in olfactory perception could be mimicked by the ICV administration of leptin in starved rats fed for 2 h a day, or that of orexin in animals fed ad libitum (Julliard et al., 2007).

However, the consequences of a chronic modification of feeding patterns have been less well documented, but may provide insight into whether the olfactory system can also elicit long-term plasticity. We therefore decided to investigate the effect of modifying the feeding schedule by comparing rats habituated to receiving a single meal for 2 h daily versus rats with access to food for 8 h daily. The effects of such a caloric restriction were measured on both food-driven olfactory behaviors and the expression of insulin and leptin receptors in the OM and OB.

Materials and methods

Animals

One month-old male Wistar rats from our breeding stock were housed under 12 h/12 h LD cycles, the lights being switched off at 09:00. The temperature in the animal house was maintained at a constant level of around 22 °C; the animals had free access to tap water, and were fed with food pellets (M25; Dietex, Saint-Gratien, France) from 09:00 to 17:00. At 6 wk old, when the olfactory system is fully mature (Saito et al., 1998), they were randomly assigned to two feeding schedules: long feeding (LF: food available from 09:00 to 17:00) or short feeding (SF: food available from 11:00 to 13:00).

A first group of rats was weighed daily and their food consumption was measured until the animals were 9 wk old, in order to validate the experimental model. These animals were then sacrificed to determine both metabolic parameters and insulin receptor levels. In a second group, behavioral experiments were performed between the ages of 9 and 12 wk before they were sacrificed for quantitative PCR (qPCR) measurements of both insulin and leptin receptors.

The rats were killed by decapitation after their mealtime during the imposed night period (15:00 for SF and 21:00 for LF rats), far away from their mealtime to minimize the bias introduced by the post-prandial peak of insulin and of the vagus-nerve olfactory pathway activation on our data. All rats were weighed prior to sacrifice. Trunk blood samples were collected via the exposed dorsal aorta, centrifuged at 3000 *g* for 30 min to collect plasma and then kept at −20 °C until assay. The OM was removed immediately and dissected on an ice-cold plate. The OB and liver were also removed immediately. The tissues were stored at −80 °C until either molecular or biochemical extractions.

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Every effort was made to minimize the number of animals used and their suffering. Euthanasia was properly conducted by skilled staff using well-maintained equipment that ensured a rapid death. C.B. holds the Individual Authorization for Performing Experiments in Animals, provided by Préfecture des Yvelines (France), according to French and European laws (agreement #78-65).

Olfactory-driven food behaviors

Two different tests were performed: the first (tea-ball test) was designed to measure locomotor activity and sniffing behavior in response to a food odor stimulus (Prud'homme et al., 2009) and the other (hidden cookie test) is currently a standard test used to determine broad olfactory acuity (Le Pichon et al., 2009). For the tea-ball test, at the beginning of the light phase, the rats were placed individually in clean cages in a test room for a habituation period of 10 h with water but no food. At the end of this period, the behavior of rats in the presence of a food odor was recorded: each rat was challenged with empty tea-balls as controls or tea-balls containing food pellets; the tea-balls were placed carefully on top of the cages, taking care not to disturb the rats; the time spent moving about and sniffing the tea-ball was recorded for 2 periods of 20 min separated by a 30 min rest period. The same experiment was then performed on the same rats after a 24 h fasting period.

For the hidden cookie test, at the end of the light phase, rats were placed in a clean cage containing a chocolate cookie buried under the bedding, and the time taken to retrieve the cookie was recorded. This test was performed twice a day per rat in LF or SF status, on 4 consecutive days. However, on the 5th day, the experiments were carried out after a 24 h fasting period.

Plasma metabolic parameters

Blood glucose and triglyceride levels were measured using commercial kits (the Glucose HK assay kit from Sigma, Saint-Quentin Fallavier, France and the triglyceride enzymatic PAPA150 kit from BioMérieux, Craponne, France) in accordance with the manufacturer's recommendations.

Insulin receptor levels

OM, liver and OB membranes from either LF or SF rats were prepared as follows: the tissues were collected and homogenized in a Teflon glass homogenizer with a four-fold volume of 1 mM $NaHCO_3$ buffer containing 20% sucrose, 1 mM ethylenediaminetetracetic acid, 1 mM PMSF and 10 μg/ml leupeptin maintained at 4 °C. The homogenates were centrifuged at 9000 *g* for 30 min at 4 °C and the resulting supernatants were further centrifuged at 105,000 *g* for 60 min at 4 °C. The resulting pellets were suspended in the insulin binding assay buffer (1 ml buffer/g homogenized fresh tissue) and stored at −80 °C. The protein concentration in the membrane suspension was determined using the method described by Peterson. The binding of ^{125}I-labeled insulin (^{125}I Tyr A14 porcine IR grade, specific activity 371 μCi/μg; NEN, Boston, MA, USA) to OM, liver and OB membranes was performed in duplicate, as previously described (Lacroix et al., 2008).

Briefly, membranes were incubated with 15 fmol of ^{125}I-insulin and serial dilutions of unlabelled bovine insulin (Sigma Aldrich) overnight at 4 °C. At the end of the incubation period, membranes were centrifugated and radioactivity of the pellets was determined in a gamma counter (LKB 1272, LKB instruments, Victoria, Australia). Specific binding was determined and Scatchard plots were analyzed with LIGAND program.

RT-quantitative real time polymerase chain reaction (RTqPCR)

Total RNAs were prepared from the OM and OB of either LF or SF rats according to the guanidium-thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987) and quantified using Agilent profile. 60 ng cDNA was obtained by the reverse transcription of 4 µg of mRNA for each sample, using Superscript™ First-Strand Synthesis, mixed with 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Villebon-sur-Yvette, France) and 300 nM of each primer complementary to either the different target genes or to ß-actin, in a total volume of 20 µl (Table 1). ß-actin was chosen as the reference based on its constant expression following food deprivation (Baly et al., 2007) and it was verified that it varied by less than 1 Ct between SF and LF rats during this study (data not shown). The reaction mixture was transferred into a 96-well optical reaction plate, sealed with appropriate optical caps and run on the ABI Prism 7900HT apparatus (Applied Biosystems) under standard conditions. All expression data were normalized to the level of ß-actin expression from the same individual sample. Quantification results are expressed either a fold activation relative to LF status or as deltaCt compared to β-actin.

Statistical analysis

Values were expressed as mean ± SEM. For behavioral data, values were compared using two-way ANOVA (SF or LF and fasted or fed) followed by the Bonferroni post-hoc test. For other measurements (body weight and food intake, metabolic parameters, metabolic expression levels, insulin receptor levels), values were compared using Student's t test. For qPCR analyses, the expression values of the different receptors for SF and LF rats were compared using one-way ANOVA followed by Tukey's post-hoc test; the effect of the feeding regimen on the level of expression of a given receptor was tested using the Mann–Whitney test. Statistical significance was taken as $P<0.05$.

Results

A short feeding period results in reductions in food intake and body weight (Fig. 1)

At the beginning of the experiments, the initial body weight of the rats was 86.5 ± 2.3 g (mean ± SEM, $n=10$). During the first 2 wk of acclimatization to the light and daily feeding rhythm, the daily food intake increased gradually to 20.22 ± 0.56 g and the weight of the rats rose to 141.6 g ± 3.8 g (Fig. 1A). The rats were then randomly distributed to either overnight long feeding (LF) or short feeding (SF) paradigms at day 13. In SF rats, shortening of the feeding time led to a significant and rapid decrease in the average daily food intake which subsequently remained stable; the mean daily food intake until the end of the experiment was 13.4 g, whereas in LF rats the mean daily food intake was significantly higher (20.4 g, $P<0.05$). This significant difference led to a daily body weight increase of about 5.6 g/day in LF rats and 2.98 g/day in SF rats ($P<0.05$); the body weight then increased linearly ($R^2=0.99$ for LF and $R^2=0.97$ for SF) in the two groups until the rats were 9 wk old (Fig. 1B). Restriction of the feeding period thus led to a less marked increase in body weight. The final weight of the SF rats was 75% of that of the LF rats (253.38 ± 5.40 g for LF versus 190.28 ± 3.34 g for SF, n = 5).

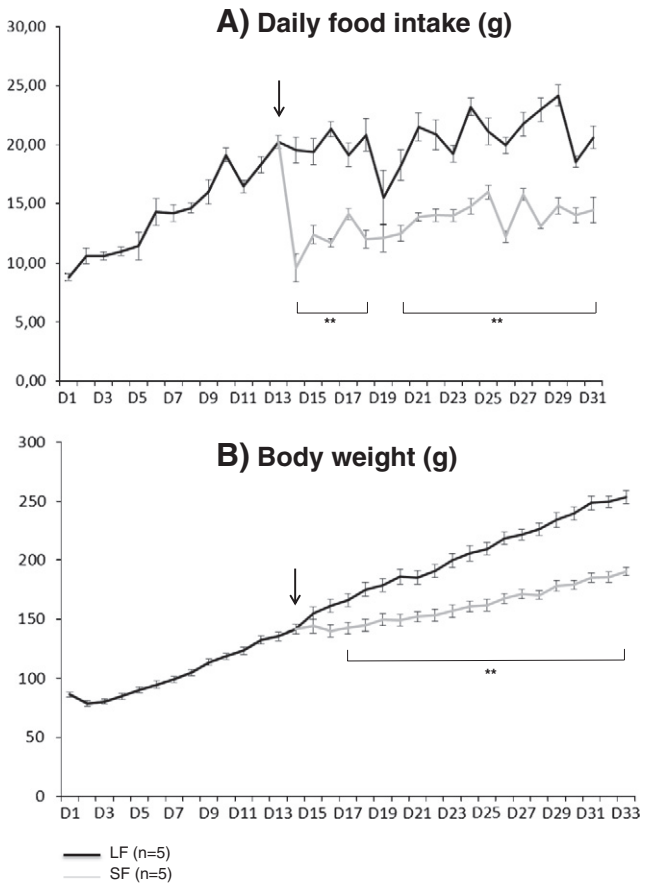

Fig. 1. Effect of food restriction on the daily food intake and body weight of SF and LF rats. Time-course of daily food intake (A) and body weight increase (B) in male Wistar rats before (ad libitum, days 1 to 14) and after restricted access to food for either 8 h per day (long feeding; LF, black line) or 2 h per day (short feeding; SF, gray lines). Values are the mean ± SEM. **P<0.01 (Student's t test).

A short feeding period results in significant modifications of blood metabolic parameters (Fig. 2)

For the determination of metabolic parameters, a second group of rats raised under the same conditions was sacrificed at 9 wk old, with a mean body weight of 261.3 ± 4.8 g for LF rats ($n=10$) and 209.9 ± 6.2 g for SF rats ($n=8$) ($P<0.05$). The restricted feeding period resulted in a 20% decrease of SF rats' body weight, as for the former group. At 9 wk old, the metabolic and endocrine status of SF (n = 13) and LF rats (n = 15) differed significantly: levels of triglycerides (0.94 ± 0.07 vs. 1.26 ± 0.09 g/l), glucose (1.52 ± 0.03 vs. 1.64 ± 0.04 g/l), insulin (1.53 ± 0.47 vs. 2.91 ± 0.28 µg/l) and leptin (2.62 ± 0.29 vs. 3.47 ± 0.30 µg/l) were significantly lower in SF rats ($P<0.05$).

Table 1
Primers for real-time PCR.

Gene name	Genbank accession number	Primers	Amplicon size
Leptin	NM_013076.3	F5′ TTCACACACGCAGTCGGTATC3′ R5′CCCGGGAATGAAGTCCAAA3′	61
Ob-Ra	AF304191.1	F5′TTTCCAAAAGAGAGCGGACAC3′ R5′AGGTTGGTAGATTGGATTCATCTGT3′	70
Ob-Rb	AF287268.1	F5′AAAGCCTGAAACATTTGAGCATC3′ R5′CCAGAAGAAGAGGACCAAATATCAC3′	70
Ins1	NM_019129.3	F5′GCCCAGGCTTTTGTCAAACA3′ R5′TCCCCACACACCAGGTACAGA3′	71
Ins2	NM_019130.2	F5′GGCTTTTGTCAAACAGCACCTT3′ R5′TCCCCACACACCAGGTAGAGA3′	66
IR-A	NM_017071.2	F5′CAGGCCATCCCGAAAGC3′ R5′GGTGTAGTGGCTGTCACATTGC3′	60
IR-B	NM_017071.2	F5′TTACCTGCACAACGTGGTTTTT3′ R5′CTCAGCACCATTGCCTGAAG3′	58
B-actin	NM_031144.2	F5′GACCCAGATCATGTTTGAGACCTT3′ R5′CACAGCCTGGATGGCTACGT3′	61

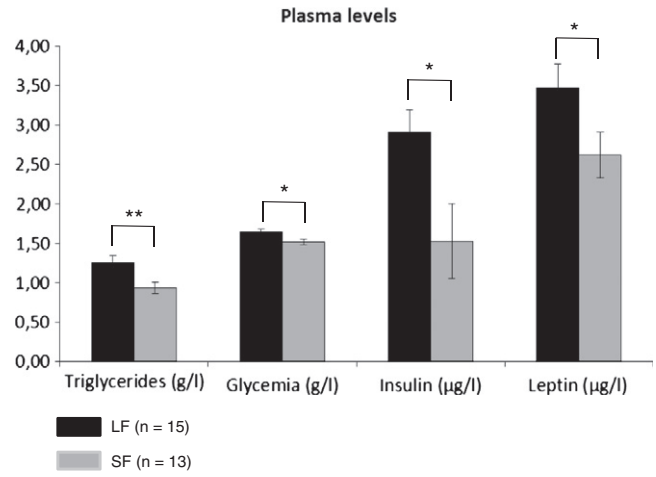

Fig. 2. Effect of food restriction on body weight and blood metabolic parameters. The histograms represent the values for blood metabolic parameters of LF and SF rats at the time of sacrifice. Data are given as mean ± SEM. **P<0.01; *P<0.05 (Student's t test).

A short feeding period results in modifications of olfactory-driven food behaviors (Fig. 3)

Between 9 and 12 wk, the rats were tested for their olfactory behavior using two different behavioral tests. We looked first at locomotor activity and sniffing behaviors induced by a tea-ball containing food pellets (or not) placed on top of the cages containing LF and SF rats ($n = 12$) (Fig. 3A). There was a great variability in the locomotor activity of the rats, some of them remaining quiet and asleep in their cages, the others being active throughout the test period, even in the presence of an empty tea-ball. The global two-way ANOVA showed that the presence of food in the tea-ball significantly increased locomotor activity ($P<0.001$) and that rats fed for 2 h a day displayed greater locomotor activity than those fed for 8 h a day ($P<0.01$). In the presence of food, as well as in the presence of an empty tea-ball, fasting significantly increased the locomotor activity of both LF and SF rats (Fig. 3A top; $P<0.01$). When the LF and SF groups were analyzed separately, SF rats were significantly more active in the presence of food, and fasted rats were more active than fed ones. In LF rats, the presence of food in the tea-ball did not modify locomotor activity when compared to the empty ball; however, fasted rats were more active under both tea ball conditions (Fig. 3A).

In the presence of an empty tea-ball, the time spent sniffing was very short (Fig. 3A, bottom). The global two-way analysis of variance showed that all groups of rats spent much more time sniffing in the presence of a food odor ($P<0.001$); there was also a significant effect of nutritional status ($P<0.05$), with an interaction. In the presence of an empty tea-ball, neither the duration of the feeding period (LF vs. SF rats, $P>0.05$) nor a 24 h fasting period had any effect. In the presence of food odor, SF rats spent significantly more time sniffing than LF rats ($P<0.001$). However, a 24 h fasting period had no effect (Fig. 3A).

We then tested the global olfactory capacities of rats by means of a hidden cookie test. The mean time spent finding the buried cookie during two consecutive tests is represented in Fig. 3B. There was a significant time effect as the rats learned the task and became more efficient at finding the cookie, but both groups behaved similarly, and a 24 h fasting period had no effect on their global olfactory performance (comparison of days 4 and 5, Fig. 3B).

A short feeding period results in modifications of the expression of insulin receptors in the olfactory system

Because the expression of insulin receptors is increased after a 48-h fasting period and insulin clearly modulates olfactory signals in the OM (Lacroix et al., 2008), we tested the effects of a chronic modification of metabolic status on the expression of insulin receptors in the olfactory system (mucosa and bulb) and in the liver as a control. As already shown, membranes from the olfactory system efficiently bound ^{125}I-insulin, and Scatchard plot analyses suggested a two-order receptor site model (not shown). For high affinity sites, the Bmax are reported in Fig. 4. In the OM, the number of binding sites was slightly decreased after 2 wk of food restriction (14.8 ± 3.2 fmol/mg protein in LF rats, 7.6 ± 1.7 fmol/mg protein in SF rats, $P = 0.09$); the feeding pattern did not modify the number of binding sites in the OB (6.85 ± 1.45 fmol/mg protein in LF rats, 6.23 ± 1.35 fmol/mg protein in SF rats). In the liver, insulin binding site number was far higher than that found in the mucosa and bulb and a 2-h feeding period for 2 wk resulted in an increase in this number (473.5 ± 61.4 fmol/mg protein in SF rats vs. 138.9 ± 44.6 fmol/mg protein in LF rats, $P<0.01$). The low level of leptin receptor expression in the OM (Baly et al., 2007), causing technical limitations on the quantification assay, prompted us not to quantify leptin receptors at the protein level, but to complete the data at the transcriptional level.

A short feeding period slightly changes transcription of the insulin and leptin receptor gene system

In order to gain further insight into the mechanisms by which the expression of insulin receptors is regulated after a long-term modification of feeding rhythm, we performed a real-time quantitative PCR analysis of mRNAs isolated from either SF or LF olfactory tissues, and quantified the expression of both leptin and insulin receptors, as previously described (Baly et al., 2007; Lacroix et al., 2008, and see Table 1 for the characterization of primers). LF and SF rats were then sacrificed at 12 wk old, with mean body weights of 301.4 ± 8.3 g and 250.1 ± 11.4 g, respectively. The relative messenger levels, expressed as a mean normalized to an endogenous reference (ß-actin) were expressed as relative quantification levels of SF versus LF rats and as a delta Ct representation (Figs. 5A and B, respectively).

In the OM, q-PCR values for insulin receptors indicated a slight decrease in both IR-B (non-significant) and IR-A ($P = 0.05$, Mann Whitney test) messenger levels in SF rats subjected to a daily 2 h-controlled mealtime. In the OB, opposite variations were observed for insulin receptors, with an almost 2-fold up-regulation of IR-B in SF rats compared to LF rats ($P = 0.09$, Mann Whitney test), and a non-significant increase in IR-A. By contrast, the levels of Ob-Ra and Ob-Rb leptin receptors were unchanged in both OM and OB tissues from SF rats when compared to LF rats (Fig. 5A).

Receptor transcript expression levels were then compared in LF and SF rats using one-way ANOVA followed by Tukey's post hoc test (Fig. 5B). In the OM and OB, the OB-Ra isoform was significantly more present than OB-Rb. In both tissues, the short form of the insulin receptor, IR-A, was also significantly more strongly expressed than IR-B. The level of expression of the IR-B isoform was found to be far lower in the OB than in the mucosa. When comparing the expression levels of insulin and leptin receptors, we showed that IR-A was significantly more present in olfactory tissues than both IR-B and the two isoforms of leptin receptors.

Overall, we showed here that the expression of leptin receptors was unaffected by the chronic food restriction in both the OM and OB of SF rats when compared to LF rats. In SF rats, insulin receptor expression was slightly decreased in the OM and increased in the OB.

Discussion

The present study demonstrates that a 2 h daily feeding period was followed by a 35% reduction in food intake and a 20 to 25% decrease in body weight, leading to significant plasma metabolic and endocrine modifications; consequently, the expression of insulin receptors in the OM and bulb was slightly changed under this type of

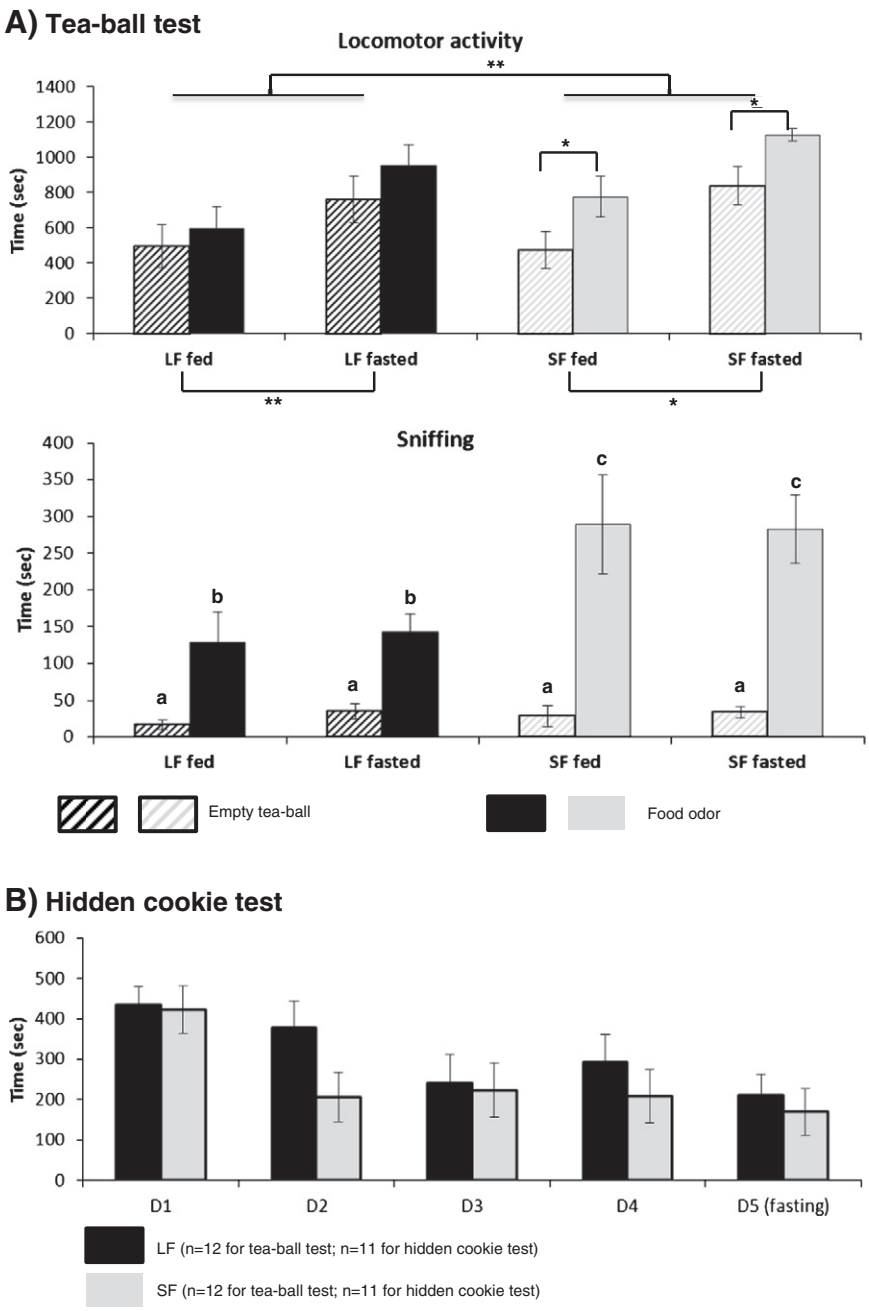

Fig. 3. Analysis of the behavioral effect of food intake restriction on SF and LF rats. A: Tea-ball tests: locomotor activity (up) and sniffing time (middle) measured during presentation for 20 min of an empty tea-ball (hatched histograms) or of food odor (solid histograms) in LF (black) and SF (gray) rats. The same test was performed in fed rats, and after a 24 h fasting period. Values are mean ± SEM ** $P<0.01$; * $P<0.05$ (Two-way ANOVA followed by Bonferroni post-hoc test). B: Hidden cookie test: the histograms represent the mean time required to find a buried cookie (two tests a day); the tests were performed for 4 consecutive days in fed rats and on the 5th day, in 24 h fasted rats. LF rats: black histograms; SF rats: gray histograms. Values are mean ± SEM.

chronic restricted nutritional state. However, these modifications affected olfactory-driven food behaviors, altering locomotion and sniffing in order to adapt food searching and olfactory signal processing to the metabolic status. These data thus reinforce the growing evidence of a link between olfaction and nutrition, through the first demonstration in non-genetically modified rodents that the olfactory system is sensitive to subtle long-term modifications of metabolic status.

Chronic food restriction achieved by manipulating the meal window has been widely used to analyze the consequences of calorie restriction on global physiological parameters such as energy metabolism, aging, brain health and neuronal plasticity, in both animal models and humans (for a review, see Mattson et al., 2003; Redman and Ravussin, 2009). It is also a widely used protocol for behavioral experiments when a consistent level of motivation is required (see the overview by Rowland et al., 2008), and has been used in numerous studies dealing with food anticipatory activity and brain oscillators entrained by food (for a review, see Silver and Balsam, 2010).

As already described in rats subjected to a 2-h feeding period (Poulin and Timofeeva, 2008) adaptation to short scheduled feeding was accompanied by a reduction in the daily food intake and a lower increment in body weight that persisted until the end of experiment, 2 to 5 wk later. At the time of sacrifice, the metabolic status of both SF and LF rats was in accordance with the findings of several other studies, where rats displayed a modification to potential mediators of the adaptive response to calorie restriction such as

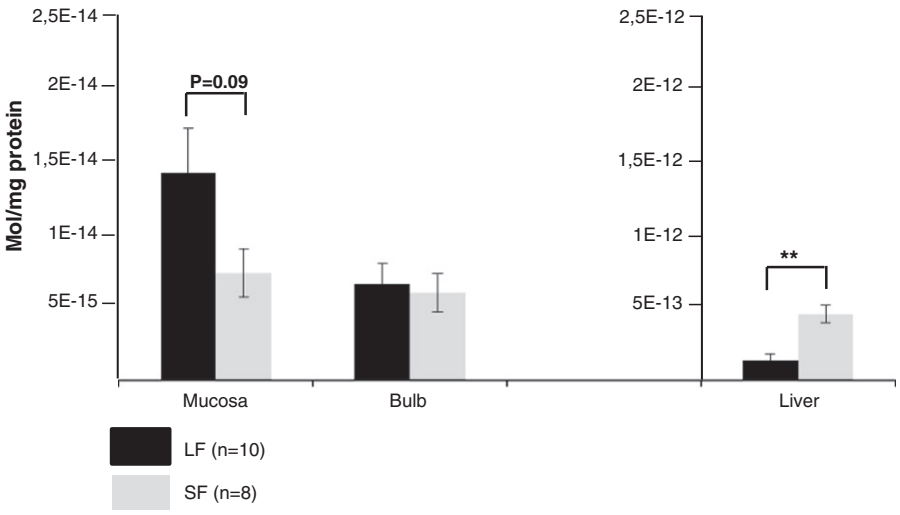

Fig. 4. Effect of food intake restriction on insulin binding capacities in different tissues from SF and LF rats. Histograms represent the insulin binding capacity (Bmax) of high affinity sites in membranes collected from the olfactory mucosa (left), olfactory bulb (middle) and liver (left) of LF (black histograms) or SF (gray histograms) rats. Values are given as mean ± SEM. **P<0.01.

triglycerides, glucose, insulin and leptin (Chiba et al., 2002; Mattson et al., 2003). Indeed, we observed that both glucose and triglyceride levels were lowered significantly by chronic food restriction.

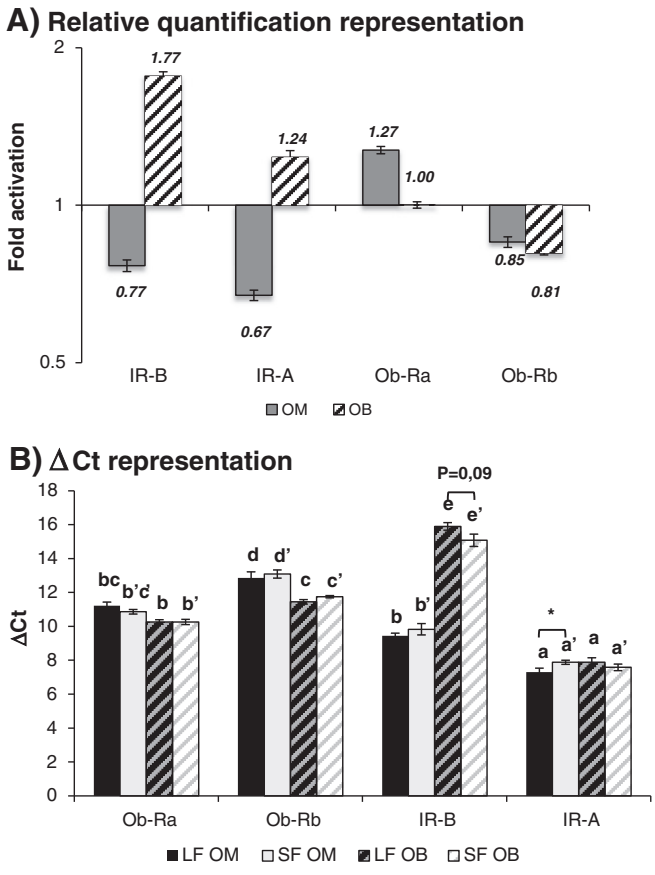

Fig. 5. Relative quantification of the expression of leptin and insulin receptors genes in olfactory tissues following food restriction. RT-PCR quantifications of insulin (IR-A and IR-B) and leptin (Ob-Ra and Ob-Rb) receptors are represented as fold activation in SF rats versus LF rats (A) or as a deltaCt representation relative to the β-actin reference gene (B) ($n = 5$ for each condition for OM; $n = 6$ for each condition for OB). Values with different superscripts differ significantly ($a \neq b \neq c \neq d \neq e$ in LF rats; $a' \neq b' \neq c' \neq d' \neq e'$ in SF rats) (one-way ANOVA followed by Tukey's post hoc test). *P<0.05 when SF and LF transcripts are compared.

Furthermore, leptinemia, which is correlated with adipose tissue mass (Saladin et al., 1995), and insulinemia, which is also considered as a long-term adiposity signal (for a review, see Havel, 2001; Moran and Ladenheim, 2011), were significantly altered by mealtime duration, despite great variations between individuals. However, a comparison between the plasma levels of triglycerides, glucose, insulin and leptin that we measured in rats fed for 2 or 8 h, during the dark phase of the day, and other published data is hazardous, because of the differences in experimental conditions: timing of the feeding period (mainly during the light phase of the day) and of sacrifice, and age of the animals at the end of the experiment. Furthermore, glucose, insulin and leptin concentrations are known to display a diurnal rhythm (for a review, see Kalsbeek et al., 2010; Cuesta et al., 2009), that can be extensively altered by food availability (Feillet, 2010). However, like Bi et al. (2003), we showed here a significant decrease in insulin and leptin concentrations in rats fed for 2 h when compared to those fed for 8 h; indeed, this reduction was far greater than that observed after an acute 48 h period of food restriction (Baly et al., 2007; Bi et al., 2003; Lacroix et al., 2008). In the rat, circulating hormonal levels, and also the expression of hypothalamic neuropeptides, are known to be differentially affected by chronic and acute food restriction (45% of the quantity consumed by control animals or 48 h of fasting) (Johansson et al., 2008; Sucajtys-Szulc et al., 2010). Our results thus suggest that such chronic food restriction differentially impacts the modulation of insulin and leptin receptor genes and olfactory functions.

Indeed, in fasted rats, the number of insulin binding sites in the OM was increased without there being any changes to IR-A and IR-B transcripts (Lacroix et al., 2008), whereas the expression of Ob-Ra and Ob-Rb leptin receptors mRNAs was also up-regulated (Baly et al., 2007); by contrast, in the OB, acute food restriction did not modify the expression of leptin receptors (Prud'homme et al., 2009), or that of insulin receptors (Badonnel, personal communication). Interestingly, regulation of the expression of insulin and leptin receptors during chronic food restriction was less marked than during studies performed after acute 48-h food starvation.

In SF rats, no significant variation in leptin receptor mRNAs was detected in either the mucosa or the bulb. However, in the same way as for insulin receptors after 48 h of fasting, discrepancies in the OM between mRNAs and protein levels were possible (Lacroix et al., 2008), but a final demonstration at the protein level was

hampered by the small quantities of receptors available. Moreover, because Ob-Ra and Ob-Rb transcripts are less abundant than IR transcripts in these tissues, faint modifications to far less strongly expressed molecules could be much more difficult to quantify.

In SF rats, the slight down-regulation of both insulin receptor transcripts is accompanied by a tendency to a down-regulation of the number of insulin binding sites in the mucosa; this regulation was not observed in the OB at the protein level, whereas at the mRNA level, the IR-B receptor was slightly up-regulated. Indeed, these observations would need to be confirmed on greater animal samples.

In the liver, contrary to what was observed in the mucosa, restricted animals displayed a significant increase in the number of insulin receptors, corresponding to the expected up-regulation of receptors after a fall in hormone levels. Indeed, the number of insulin binding sites is due to both IR-A and IR-B receptor isoforms; both isoforms are expressed equally in the olfactory system, whereas the liver predominantly expresses the long form of the receptor (Lacroix et al., 2008).

The inverse variations in the number of insulin binding sites in the OM and liver of food-restricted rats could be due to either a different regulation of IR-A and IR-B isoforms, or to the local production of insulin by OM (Lacroix et al., 2008).

Therefore, the faint modifications to insulin and leptin plasma concentrations arising from chronic food restriction affected insulin but not leptin receptor levels. It is clear that long-term restriction results in a metabolic neuroendocrine adaptation which differs from that seen with short-term fasting, and could therefore have a different impact on peripheral tissues (Chiba et al., 2002; Lecoultre et al., 2011).

A growing body of evidence shows that the OM and bulb are functional targets for insulin and leptin, since they interfere with olfactory processing. Using electrophysiological approaches, it has been shown that both hormones are able to decrease the amplitude of the neuronal response to odorants after their direct application to the OM (Lacroix et al., 2008; Savigner et al., 2009). In the OB, insulin modifies the odor response of mitral cells in anesthetized rats (Cain, 1975) and changes the response of mitral cells *in vitro* in a sensory-dependent manner (Fadool et al., 2000). After exposure to food odor, leptin decreases the c-Fos response of mitral and granular cells (Prud'homme et al., 2009). Both the OM and bulb have thus been identified as functional targets for insulin and leptin. However, under the experimental conditions described above, i.e. fasting or direct hormonal application, an acute and massive alteration of hormonal levels resulted. To our knowledge, little evidence is available at present regarding the functional effects of chronic metabolic modifications in the OM and OB.

Nevertheless, we demonstrate here for the first time that these metabolic changes affect not only circulating insulin and leptin levels and the expression of insulin receptors in the olfactory system, but also clearly modulate some olfactory-driven food behaviors.

We chose to induce chronic metabolic changes in rats by manipulating the duration of feeding. Many studies have shown that when food availability is restricted to a single period at a fixed time of the day, rats adapt to this condition within a few days by increasing their food-seeking activity during preceding hours (Challet and Mendoza, 2010; Mistlberger, 1994; Stephan, 2002). During our study, the behavioral tests were performed at the end of the light phase of the day, when the rats were still in their resting period, but at a time when food anticipatory activity started (Gunapala et al., 2011). Both groups of rats displayed a high level of locomotor activity, even in the presence of an empty tea-ball, which could have been due to this anticipatory pattern of activity; however, SF restricted rats were globally more active, and food odor was only followed by an increase in locomotion in this group. As a control, a 24 h fasting period increased locomotor activity in both groups of rats. Taken together, these results suggest a stronger motivation for food seeking in restricted rats. Few data are available on the molecular determinants of food anticipatory activity. In a recent study using knockout orexin- or leptin-deficient mice, Gunapala et al. (2011) showed that orexin deletion decreased food anticipatory activity, while that of leptin reduced the walking and rearing components of this activity, which was in light with our findings.

A negative energy balance has been reported to enhance food palatability (Cameron et al., 2008) and food hoarding behavior (Shizgal et al., 2001), thus demonstrating the diversity and complexity of adaptive responses to overcome energy deficiency. Sniffing behavior is an odor sampling strategy coordinated with an attentional state and locomotion (for a review, see Wachowiak, 2011). Our results showed very little sniffing behavior in the presence of an empty tea-ball. But when it was filled with food, sniffing increased in SF rats when compared to LF rats. These results suggest a stronger motivation (interest) for food seeking in restricted rats, as already observed in ad libitum rats after a 48 h fasting period (Prud'homme et al., 2009).

The experimental modification of metabolic peptides/hormonal profile leads to modulation of olfactory behaviors: orexins, ghrelin and leptin are some of the known molecular determinants of food-induced locomotor and sniffing behavior (Prud'homme et al., 2009; Tong et al., 2011). Tea-ball tests performed in ad libitum fed rats revealed very little locomotor and sniffing activity, even in the presence of tea-balls containing food pellets (Prud'homme et al., 2009). The administration of orexin receptors antagonists or leptin in 48 h fasted rats was able to mimic such a behavioral fed status. No data are available yet regarding a role for insulin in such food-driven behaviors.

We used the hidden cookie test to test the olfactory performance of both SF and LF rats. SF rats found the cookie slightly more rapidly than LF rats; in neither group did a 24 h fasting period have any effect. This test is widely used to investigate olfactory-mediated behavior in rodents, and was indeed employed to demonstrate that leptin-deficient mice are much more rapid in finding a hidden cookie (Getchell et al., 2006). This might suggest that the decrease in insulin and leptin plasma levels in SF rats compared to LF rats was too subtle to influence global olfactory performance.

Conclusions

Taken together, our results demonstrate for the first time that chronic modifications to food intake, leading to slight changes in insulin and leptin levels, induced an adaptive response in olfactory-driven food seeking, without clear modifications of olfactory performance. This adaptive response may occur at the level of the olfactory system, mucosa and bulb, and also in many other regions of the brain that are sensitive to insulin and leptin. Finally, since caloric restriction results in an increased motivation for food in rats, it could be important to evaluate the food-driving olfactory behaviors in overweighed humans under caloric-restricted diets, where effects on subjective hunger have been described (Redman and Ravussin, 2011).

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