

Transcription Profile Analysis Reveals That OBP-1F mRNA Is Downregulated in the Olfactory Mucosa Following Food Deprivation

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Abstract

Neuroanatomical data show that olfactory mucosa (OM) is a possible place for interactions between nutrition and smell. A combination of differential display mRNA analysis together with a macroarray screening was developed to identify transcripts that are differentially expressed in rat OM following food deprivation. Using this method, backed on a stringent statistical analysis, we identified molecules that fell into several Gene Ontology terms including cellular and physiological process, signal transduction, and binding. Among the 15 most differentially expressed molecules, only one was upregulated, but 14 were downregulated in the fasted state among which was, unexpectedly, odorant-binding protein 1F (OBP-1F). Because of its potential relevance to olfactory physiology, we focused our further analysis on OBP-1F using *in situ* hybridization, quantitative polymerase chain reaction, and western blot analysis. OBP-1F was highlighted in the lateral nasal glands, but its expression (mRNA and protein) did not change following food deprivation. Only the minor fraction of OBP-1F mRNA expressed by the OM itself was downregulated following 48 h fasting. Altogether, our results suggest that the fine transcriptional control of OBP-1F in the OM following food deprivation could be efficient only at the local level, close to its site of secretion to participate in the perireceptor events of the olfactory signal reception.

Key words: food intake, lateral nasal glands, macroarray, neurotransmission, odorant-binding protein, q-PCR

Introduction

Olfaction is a major chemical sense involved in several mammalian behaviors such as food intake, social interactions, or reproduction. The relations between olfaction and nutrition are multiple and have been hypothesized for a long time (Pager 1974). Olfactory stimuli induce metabolic modifications partly through the regulation of the digestive tract activity. Food odors influence circulating glucose and insulin levels and induce saliva secretion and gastric contractions (Mattes 1997). Neuroanatomical data show that olfactory mucosa (OM) and olfactory bulb (OB) are possible places for these interactions: the hypothalamic structures, which analyze the metabolic signals and in return control food intake and energy expenditure, project themselves directly to

the OB (Hardy et al. 2005). In the OM, recent studies show the presence of many peptides and their receptors related to feeding status: orexins (Caillol et al. 2003) and leptin (Baly et al. 2007), some being locally synthesized and the others being brought by the general circulation. Moreover, an orexigenic peptide, orexin A, reinitializes a starving status in a fed rat (Sakurai et al. 1998) and modulates mitral cells response of the OB (Hardy et al. 2005). If these neuroanatomical and behavioral data support a possible interaction between smell and nutrition, few molecular analyses were undertaken to extend these results at the molecular level.

Olfaction is based on the reception of odorant molecules reaching the OM. The OM is located in the posterior part

of each nasal fossae (Nef 1998); it consists of an olfactory epithelium (OE) lying on the lamina propria (LP). The OE is composed of olfactory sensory neurons (OSNs) surrounded by supporting cells and of basal stem cells that ensure the continuous renewal of the neurons throughout life. Each bipolar sensory neuron extends a single dendrite with a dendritic knob bearing ciliae, where the olfactory receptors are expressed (Pelosi 1996). The odorant molecule reaches the OSN ciliae through a thin layer of mucus covering the OM. The mucus is produced at the surface of the OE by mucous secretory cells and by subepithelial glands of the LP. Major protein components of the mucus are the odorant-binding proteins (OBPs), which are members of the lipocalin family (Flower et al. 1993; Pelosi 1994; Briand et al. 2000). Among the 3 described rat OBPs, OBP1 is synthesized by the lateral nasal gland (LNG), one of the largest nasal glands in rats, located in the posterior area of the nose, just ventral and anterior to the OM (Pevsner et al. 1988). It is secreted in high amounts via a long duct to the tip of the nose to be atomized as watery secretions. Precise roles of OBP in early olfactory perireception events remain unclear.

The mucus composition, the renewal, the differentiation, and death of the cellular populations of the OM are continuously under the control of nervous and hormonal regulations. Not only do these regulations allow an accurate transmission of the olfactory signal but they can also modulate this signal according to the physiological status of the animal, such as the nutritional status.

In this paper, we studied the putative link between these 2 important physiological functions through a transcriptional profile analysis of OM of Wistar rats. We investigated the gene expression patterns using an RNA differential display analysis (ddmRNA) followed by a high-density filters (macroarray) measurement, between normally fed (fed) or 48-h food-deprived (fasted) rat OM. We identified several molecules exhibiting a modified level of expression between the 2 nutritional conditions. A single molecule, the odorant-binding protein 1F (OBP-1F) (Briand et al. 2000), was chosen for further investigations because of both its large differential expression between the 2 nutritional conditions and its broad implication in the olfactory function.

Materials and methods

Animals, diet, and experimental design

Male Wistar rats, 10–12 weeks old, from our breeding stock were housed in 12:12 h light/dark cycles with free access to food and water. All experiments were conducted according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number and the suffering of rats. Experimental conditions were based on the use of either ad libitum fed (fed) or 48-h food-deprived (fasted) rats, a restriction protocol currently used in experiments studying central effect of

food deprivation (Bariohay et al. 2005). Animals were killed by carbon dioxide asphyxiation and sacrificed by decapitation 2h after the beginning of the light phase. The OM and LNG (setting down on a cartilaginous structure at the anterior part of the nose, just underneath of the OM) were quickly removed and dissected on an ice-cold plate. Nasal OM as isolated was devoid of LNG.

Independent groups of fed or fasted rats were used for ddmRNA screening ($n = 3$, for each nutritional status), macroarray experiments ($n = 4$, for each nutritional status), in situ hybridization (ISH) ($n = 3$, fed rat), and western blots ($n = 5$, for each tissue and nutritional status). For real-time polymerase chain reaction (PCR), samples of LNG ($n = 5$ for each nutritional status) and OM ($n = 5$ for each nutritional status) were collected from brother rats and prepared as described below.

Total RNA isolation

Total RNAs were prepared from OM and LNG of fasted or fed rats following the guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987) and then DNase-I treated before quantification using spectrophotometry (macroarray) or Agilent profile (quantitative q-PCR).

For membrane screening, 50 µg of total RNA was enriched in mRNA using Clontech Atlas Pure Total RNA Labeling System, according to the manufacturer's instructions.

For the ddmRNA strategy, OM mRNA samples were pooled from 3 fed and 3 fasted rats, DNase-I treated and reverse transcribed following the manufacturer's recommendations using the Delta Differential Display kit (Clontech, Saint-Germain-en-Laye, France). Briefly, PCRs using labeled [$\alpha^{33}\text{P}$] dATP were performed using the anchor primer in combination with series of selected primers in conditions of low stringency. Amplified cDNA of fasted or fed samples were then resolved in adjacent lanes of a high resolution 15% polyacrylamide gel, and differentially expressed bands ($n = 88$) were excised from the gel, eluted, cloned into pCR2.1-TOPO plasmid (TA cloning kit, Invitrogen, Cergy-Pontoise, France), transformed in *Escherichia coli* before sequencing (Genome Express, Paris, France), and identified by Blast against nucleotide or translated and blasted against protein databases.

Constitution of the macroarray

Each array contained 203 molecules spotted in duplicate, obtained either from the differential display screening ($n = 88$, see above) or from a set of 94 genes involved in neurotransmission amplified from rat brain cDNA (provided by Dr Potier, ESPCI, Paris) together with samples of interest involved in food intake regulation or olfactory functions ($n = 21$). The bacterial clones are available under request at the CRB GADIE (francois.piumi@jouy.inra.fr).

The cDNA inserts were amplified, purified, quantified, and spotted in duplicate on Hybond N+ nylon membranes

(8 cm × 12 cm, Amersham Biosciences, Orsay, France) using the “Qpix” robot (Genetix, Hampshire, UK). After spotting, the DNA was denatured (NaOH 0.5 M, NaCl 1.5 M; 5 min), neutralized (Tris 0.5 M, NaCl 1.5 M; 5 min), and fixed onto the membrane (2 h, 80 °C). The membranes were washed with 2× sodium saline citrate (SSC), air dried, and stored at room temperature until use.

Functional information about encoded proteins was gathered from comparisons between these sequences and the rat or mouse Gene Index (Rat Release 13.0 as 10 May 2004 and Mouse release 15.0 as 17 February 2005, <http://www.tigr.org>). The Institute for Genomic Research gene indices provided links from TC (Tentative Consensus sequences) to Gene Ontology (GO) terms. A comprehensive scheme of the constituted membrane is depicted in Figure 1. The complete list of gene fragments is given in Table 1.

Synthesis of [$\alpha^{33}\text{P}$]-labeled cDNA target

One microgram of OM mRNA isolated from 8 rats ($n = 4$ for each experimental conditions) was reverse transcribed using Superscript First-Strand Synthesis System for RT-PCR Kit (Invitrogen) with 500 ng of hexamers (Promega, Charbonnières, France) and 50 U Superscript II (Invitrogen) in the presence of 10 mM of each dCTP, dGTP, and dTTP and [$\alpha^{33}\text{P}$] dATP (50 μCi).

Hybridization and posthybridization processing

Prehybridization was carried out in ExpressHyb Hybridization Solution (Clontech) during 30 min at 65 °C. Each complex target was denatured in boiling water for 5 min and hybridized in parallel with 8 distinct arrays overnight at 65 °C in rotating tubes. After a series of washing steps with high stringency, the hybridized arrays were exposed to a phosphor screen that was scanned using FLA 3000 system (Fujifilm, Düsseldorf, Germany). Spots treatment and signal quantification were done using AIDA software (Fujifilm). As expected, controls corresponding to water and vegetal-specific spots were negative.

Data analysis

Individual spot's intensities were corrected for background level and normalized for differences in probe labeling using the average intensity of spots. Changes in gene expression were calculated by the ratio of the spot intensity in fasted versus fed experimental conditions. The ratios (fold-factor) were independently calculated for each experimental replication, and genes whose expression ratio was more than twice or less than half between fed and fasted conditions were selected as candidates. The differential expression of candidates was validated by statistical analysis using 2 different algorithms in “R” language (Team 2004). A first

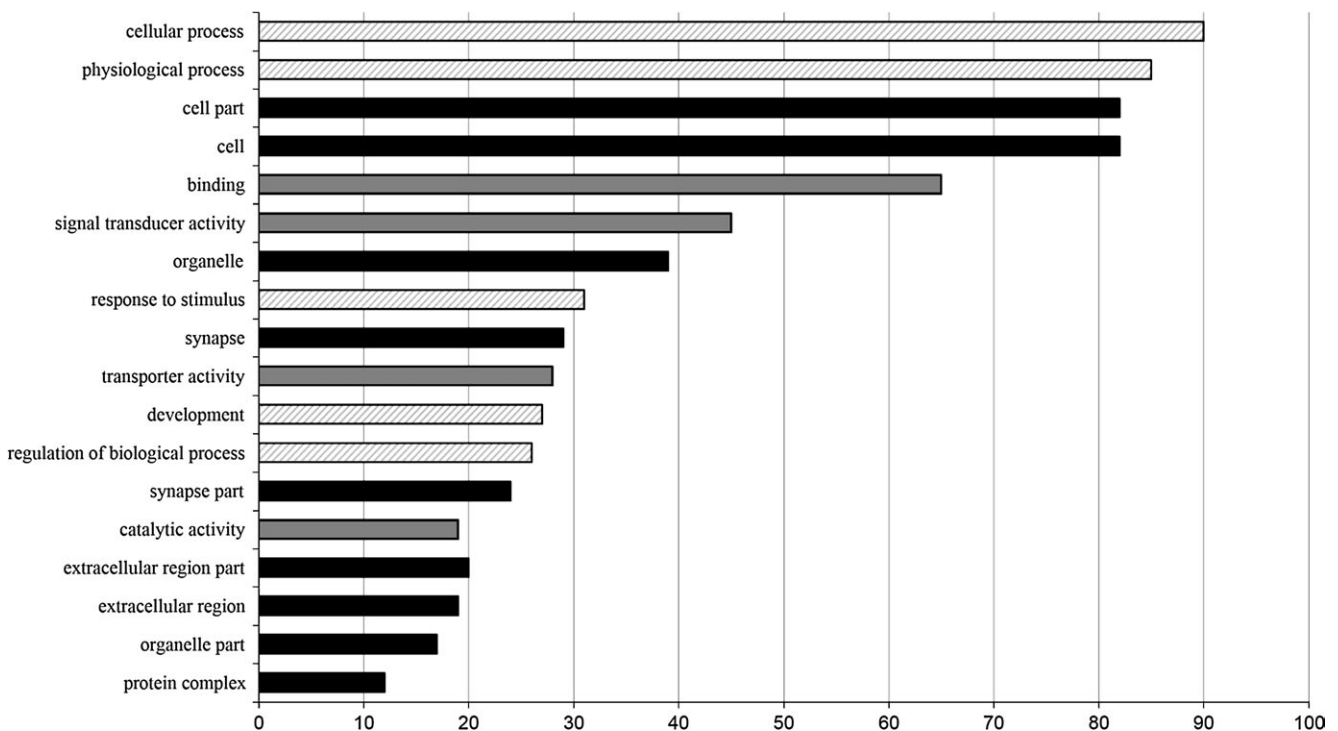


Figure 1 GO terms description of the macroarray. Almost half of the macroarray was described by GO terms. Biological processes (hatched), cellular components (black), and molecular function (gray) are identified within the 92 unique sequences of the array, which were found in the Rat or Mouse Gene Index with a link to a GO term. Only the terms representing >10 cDNAs at indent 2 are illustrated. In addition, this array contained 133 nonoverlapping sequences, which were either identified but not annotated (117) or unidentified (16).

Table 1 Complete list of gene fragments spotted on the macroarray

Accession no.	Description	Accession no.	Description
NM_018739	Mouse PAP-1	AC007362	Homo RP11-150F11
nd	nd	U63720	Mus apoptotic protease CPP32
NM_012518	Rattus calmodulin III	AL732311	Mouse DNA clone RP23-125H15
L20821	Rattus syntaxin 4	AL136457	Homo clone RP5 1015K11
AF225961	RatRhoGEFGTRAP48	AC106947	Rattus BAC CH230-19C12
L25108	Mouse LIS	AK005594	Mus progr. cell death 10
nd	nd	M64393	Rattus 3 alpha HSD
AJ238278	Rattus CRM1 exportin	NM_022310	Rattus heat shock 70-kDa protein 5 (Hspa5)
NM_022329	Mus IFN alpha responsive protein	AF338323	Mus hyaluronoglucosaminidase 1 (Hya1)
NM_013043	Rattus TGF beta-induced	AC092530	Rattus clone rp32-28p17
AB012232	Rattus NFI-B3	BX883042	Rattus CMH
nd	nd	M58040	Rattus transferrin receptor
NM_017006	Rattus G6PDH	XM_229225	Rattus bromodom adjacent to zinc finger domain
NM_017037	Rattus peripheral Myelin (Pmp22)	NM_025560	Mus Riken cDNA
AK010684	Mus ES cell cDNA	S39901	Rattus nucleoside diP kinase
AC015983	Homo RP-11-560I19	AF121344	Mus tetraspanin Tspan5
nd	nd	nd	nd
NM_019289	Rattus actin-related prot complex 1b	AL807395	Mouse DNA clone RP23-81P12
NM_013669	Mouse synaptosomal-associated prot	XM_575093	Rattus similar to lipocalin 11
NM_001000932	Olfactory receptor Mouse/Rattus	U63315	Rattus 25-Dx
AL844603	Mouse clone RP24-334I20	NM_009211	Mus SWI/SNF Smarcc1
AC019063	Homo BAC RP11-144H20	AC010517	Homo clone LLNLR-255F4
AF259072	Rattus T-cell receptor	XM_002665	Homo cys-rich motor neuron1 (CRIM1)
XM_007462	Homo hypot pro FLJ11274	M27925	Rattus synapsin 2a
X60661	Rattus RYD5	NM_138903	Rattus OBP-1F
AF102857	18S rRNA	NM_031027	Rattus diHpyrimidine DHG
U73525	Rattus thioredoxin	D25543	Rattus golgi-associated prot GCP360
AC068952	Mouse chrm2 ct7-254f1	BX571885	Mouse DNA clone RP23-17O21
AF194970	Rattus LEK1	nd	nd
AF279286	Rattus bcl-X short form	NM_024407	Rattus similar to NADH-DH (ubiquinone) Fe-S
nd	nd	AY360148	Mus musculus lipocalin 13 (Lcn13)
NM_013179	Rattus prepro-orexin mRNA	nd	nd
NM_022708	Rattus PRL- inducible prot (PIP)	AF221952	Rattus mu protocadherin
NM_012664	Rattus synaptophysin	AB001581	Rattus DNA helicase p50
AL731854	Mouse DNA sequence from clone RP23-120A20	AC145554	Mus musculus BAC clone RP23-217E22
AC122118	Mus musculus clone RP24-225P17	AK122246	Rattus similar to mKIAA0319 protein
nd	nd	NM_145090	ADP-ribosylation fact GTPase activating prot 1
nd	nd	AK007393	Rattus KIAA1536 protein

Table 1 Continued

Accession no.	Description	Accession no.	Description
AL671117	Mouse DNA clone RP23-278L4 sur chrM X	NM_017281	Proteasom ss-alpha 4
AK004913	Mus liver cDNA	M23984	Rattus H+/P symporter
RMN26926	Rattus olfactory marker protein (OMP)	NM_012001	Rattus similar to Cop9 complex subunit 4
AF094685	SOS2 gene partial	M23674	Rattus AP50-associated Clathrin-coated vesicles
nd	nd	AM258959	Rattus norvegicus mRNA for ZH8 gene
NM_012663	Vesicle-associated membrane protein 2 (Vamp2)	V01270	Rattus 28S +18S + 5, 8S sRNA
NM_017101	Rattus peptidylprolyl isomerase A (Ppia)	NM_001013032	Neuropeptide Y receptor Y1B
NM_012596	Lept L6 : leptin receptor long form	NM_012869	Neuropeptide Y receptor Y5
AC118487	Rattus BAC CH230-285C2	NM_013064	Rattus hypocretin (orexin) receptor 1 (Hcrtr1)
X60659	Rattus RYF3	NM_013074	Rattus hypocretin (orexin) receptor 2 (Hcrtr1)
XM_232220	Rattus YY1 binding protein	nd	PRPSC : ovine prion protein
nd	Topo vector	nd	nd
nd	nd	NM_053019	Rattus AVP receptor 1A (Avpr1a), mRNA
NM_013076	Leptin mRNA	NM_115041	CG03 : cytochrome
NM_013001	Rattus paired box gene 6 (Pax6), mRNA	nd	nd
NM_012616	Rattus olfactory marker protein (Omp), mRNA	NM_053565	Rattus SOCS3
NM_017009	Rattus glial fibrillary acidic protein		
NM_017261	Gria2 : glutamate receptor, ionotropic AMPA2 (alpha 2)	X66974	Calretinin : cytosolic calcium-binding protein
NM_012956	Gabrb1 : Gammagamma-aminobutyric acid receptor, ssu beta 1	NM_183326	Gabra 1 : gamma-aminobutyric acid receptor, ssu alpha 1
NM_022225	Htr1b : 5-hydroxytryptamine (serotonin) receptor, ssu 1B	NM_017078	Chrna5 : nicotinic acetylcholine receptor, ssu alpha 5
XM_224626	CHAT : choline acetyl transferase	NM_017191	Adra1a : adrenergic receptor, ssu alpha 1a
NM_017241	Grik1 : glutamate receptor ionotropic, kainate 1	NM_012611	Nos2 : nitric oxydesynthase 2, inducible
NM_017011	Grm1 : metabotropic glutamate receptor 1 (mGluR1)	NM_012573	Grin2a : glutamate receptor ionotropic, NMDA 2A
NM_017065	Gabrb3 : gamma-aminobutyric acid receptor, ssu beta 3	NM_022202	Grm8 : metabotropic glutamate receptor 8 (mGluR)
NM_052806	Chrnb4 : nicotinic acetylcholine receptor, ssu beta 4	NM_024354	Chrna4 : nicotinic acetylcholine receptor, ssu apha 4
NM_017250	Htr2b : 5-hydroxytryptamine (serotonin) receptor, ssu 2B	NM_017362	Chrm5 : cholinergic receptor, muscarinic 5
NM_138506	Adra2c : adrenergic receptor, ssu alpha 2c	NM_024365	Htr6 : 5-hydroxytryptamine (serotonin) receptor, ssu 6
NM_012768	Drd5 : dopamine receptor 5	NM_013108	Adrb3 : adrenergic receptor, ssu beta 3
X02341	Vip : polypeptide intestinal vasoactif	NM_022499	Pva : parvalbumine
XM_001062001	Grm3 : metabotropic glutamate receptor 3 (mGluR)	Y07503	Penk1 : preproenkephalin 1
NM_080586	Gabrg1 : gamma-aminobutyric acid receptor, ssu gamma 1	AF334587	Gabra2 : gamma-aminobutyric acid receptor, ssu alpha 2
NM_017254	Htr2a : 5-hydroxytryptamine (serotonin) receptor, ssu 2A	NM_022930	Chrna9 : nicotinic acetylcholine receptor, ssu alpha 9
NM_012740	Th : tyrosine hydroxylase	NM_016991	Adra1b : adrenergic receptor, ssu alpha 1b
NM_019309	Grik2 : glutamate receptor ionotropic, kainate 2	NM_031608	Gria1 : glutamate receptor ionotropic, AMPA1
XM_343470	Grm2 : metabotropic glutamate receptor 2 (mGluR)	NM_012574	Grin2b : glutamate receptor ionotropic, NMDA 2B
NM_183327	Gabrg2 : gamma-aminobutyric acid receptor, ssu gamma 2	NM_080587	Gabra4 : gamma-aminobutyric acid receptor, ssu alpha 4

Table 1 Continued

Accession no.	Description	Accession no.	Description
NM_080773	Chrm1 : cholinergic receptor, muscarinic 1, CNS	NM_057184	Chrna6 : nicotinic acetylcholine receptor, ssu alpha 6
NM_012765	Htr2c : 5-hydroxytryptamine (serotonin) receptor, ssu 2C	NM_012585	Htr1a : 5-hydroxytryptamine (serotonin) receptor, ssu 1A
U79031	Adra2d : adrenergic receptor, ssu alpha 2d	NM_022938	Htr7 : 5-hydroxytryptamine (serotonin) receptor, ssu 7
X56306	Subst P : substance P	NM_012546	Drd1a : dopamine receptor D1A
NM_012829	Cck : cholecystokinin	NM_031984	Calb 1 : brain calbindin-d28K (CaBP28K)
NM_017012	Grm5 : metabotropic glutamate receptor 5 (mGluR)	NM_139326	Pomc1 : pro-opiomelanocortin-alpha
NM_024370	Gabrg3 : gamma-aminobutyric acid receptor, ssu gamma 3	NM_017069	Gabra 3 : gamma-aminobutyric acid receptor, ssu alpha 3
NM_012853	Htr4 : 5-hydroxytryptamine (serotonin) receptor, ssu 4	NM_019297	Chrb2 : nicotinic acetylcholine receptor, ssu beta 2
NM_052799	Nos 1 : nitric oxydesynthase 1 (neuronal)	NM_017140	Drd3 : dopamine receptor 3
NM_181373	Grik3 : glutamate receptor ionotropic, kainate 3	NM_032990	Gria3 : glutamate receptor ionotropic, AMPA3
NM_022666	Grm4 : metabotropic glutamate receptor 4 (mGluR)	NM_012575	Grin2c : glutamate receptor ionotropic, NMDA 2C
NM_017289	Gabrd : gamma-aminobutyric acid receptor, ssu delta	NM_017295	Gabra5 : gamma-aminobutyric acid receptor, ssu alpha 5
NM_031016	Chrm2 : cholinergic receptor, muscarinic 2, cardiac	NM_012832	Chrna7 : nicotinic acetylcholine receptor, ssu alpha 7
NM_024394	Htr3a : 5-hydroxytryptamine (serotonin) receptor, ssu 3A	NM_012852	Htr1d : 5-hydroxytryptamine (serotonin) receptor, ssu 1D
NM_012701	Adrb1 : adrenergic receptor, ssu beta 1	NM_012547	Drd2 : dopamine receptor 2
NM_012572	Grik4 : glutamate receptor ionotropic, kainate 4	NM_012563	Gad2 : glutamic acid decarboxylase 2
NM_012659	Smst : somatostatine	NM_017007	Gad1 : glutamic acid decarboxylase 1
NM_031040	Grm7 : metabotropic glutamate receptor 7 (mGluR)	NM_021841	Gabra6 : gamma-aminobutyric acid receptor, ssu alpha 6
NM_133420	Chrna2 : nicotinic acetylcholine receptor, ssu alpha 2	NM_012527	Chrm3 : cholinergic receptor, muscarinic 3, cardiac
XM_341111	Htr5b : 5-hydroxytryptamine (serotonin) receptor, ssu 5B	NM_017008	Gapd : glyceraldehyde 3 phosphate dehydrogenase
NM_021838	Nos3 : endothelial constitutive nitric oxydesynthase	NM_017263	Gria4 : glutamate receptor ionotropic, AMPA4
NM_017262	Grik5 : glutamate receptor ionotropic, kainate 5	NM_022797	Grin2d : glutamate receptor ionotropic, NMDA 2D
NM_022920	Grm6 : metabotropic glutamate receptor 6 (mGluR)	NM_012957	Gabrb2 : gamma-aminobutyric acid receptor, ssu beta 2
NM_052805	Chrna3 : nicotinic acetylcholine receptor, ssu alpha 3	NM_133597	Chrb3 : nicotinic acetylcholine receptor, ssu beta 3
XM_345403	Chrm4 : cholinergic receptor, muscarinic 4, cardiac	L05596	Htr1e : 5-hydroxytryptamine (serotonin) receptor, ssu 1E
NM_013148	Htr5a : 5-hydroxytryptamine (serotonin) receptor, ssu 5A	NM_138505	Adra2b : adrenergic receptor, ssu alpha 2b
NM_012492	Adrb2 : adrenergic receptor, ssu beta 2	NM_012944	Drd4 : dopamine receptor 4
NM_012614	NPY : neuropeptide Y	X53501	Thp : tryptophan hydroxylase

For each sequence, the reference accession numbers and description are specified. The macroarray contain molecules obtained from the differential display screening ($n = 88$, white), samples of interest involved in food intake regulation, or olfactive functions ($n = 21$, pale gray) and molecules from a set of neurotransmitters ($n = 94$, dark gray). Molecules in bold type are those that are found as being regulated by the nutritional status. nd: sequence with no match in database.

program determined whether the effective gene selection could have been the result of a random selection. The second one computed the probability, for each candidate, that the expression ratio between the 2 nutritional conditions was randomly distributed. The level of differential expres-

sion and the resulting statistical probabilities (P -values) were assigned to each candidate. The expression level between duplicate spots from the membranes hybridized with targets made from the same nutritional status was always highly correlated ($r > 0.99$).

RT-real-time qPCR

cDNA (60 ng) was obtained by reverse transcription of 1 µg of mRNA for each sample, using Superscript First-Strand Synthesis and mixed with 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 300 nM from each primer complementary either to OBP-1F (forward primer: 5'-CAAGTGTCTGTGGGCACCAA-3'; reverse primer: 5'-GCTGGCTGAGGAATATAATATTCCA-3'; PCR product = 63 bp) or β-actin (forward primer: 5'-GACCCAGATCATGTTTGAGACCTT-3'; reverse primer: 5'-CACAGCCTGGATGGCTACGT-3'; PCR product = 61 bp) in 20 µl total volume. β-Actin was chosen as the reference based on its constant expression following food deprivation. The reaction mixture was finally transferred into a 96-well optical reaction plate, sealed with appropriate optical caps, and ran on the ABI Prism 7900HT (Applied Biosystems) apparatus under standard conditions. Standard controls of both specificity and efficiency of the qPCR assays were performed. All expression data were normalized to β-actin expression level from the same individual sample. All results are given as mean ± SEM. Comparison between groups was made using Mann-Whitney test. Statistical significance was taken as $P < 0.05$.

Tissue preparation and ISH

ISH was carried out on OM sections from 2-month-old normally fed Wistar rats. After deep anesthesia (intraperitoneal injection of pentobarbital, Sanofi Synthelabo, France), 3 adult rats were perfused transcatheterially with 200 ml saline and then with 300 ml of a freshly prepared fixative solution of 4% PFA in PBS. Bones of the skull were discarded from the lateral and dorsal walls of the nose, and the nasal mucosa was removed as a block. Tissues were postfixed in the same fixative for 3 h at room temperature, cryoprotected with sucrose (30%), and cut in a cryostat in serial anteroposterior nasal mucosa sections of 14 µm thickness (Figure 2).

OBP-1F mRNA probe was obtained after *in vitro* transcription (IVT) of the recombinant 2.1 TOPO plasmid (Invitrogen) containing the PCR product generated after amplification using customized random primers (Delta Differential Display kit, Clontech) and corresponding to a fragment of rat OBP-1F sequence (NM_138903; position 462–756). The sense and antisense probes were synthesized after plasmid linearization and IVT using T7 or Sp6 RNA polymerase in the presence of digoxigenin-dUTP (DIG-dUTP) using DIG RNA labeling kit (Roche, Meylan, France) according to manufacturer's recommendations. Before hybridization, the slides were treated with proteinase K (10 µg/ml; 5 min), triethanolamine (0.1 M; 10 min), and anhydrous acetic acid (2.6%). Sections were dehydrated by successive baths in ethanol with increasing concentration (50–100%). The labeled probes (0.1 µg/µl) in hybridization mix were denatured beforehand at 68 °C during 10 min and hybridized overnight at 55 °C. After 3 washes in 2× SSC for 30 min each, RNase A treatment (20 µg/ml) for

30 min, immunodetection was performed using DIG Nucleic Acid Detection kit (Roche) following supplier's recommendations. Revelation time was 10 min for section 1, 40 min for section 2, and 5 h for sections 3 and 4. Slides were mounted in Vectashield (Vector AbCys, Paris, France).

Phase-contrast images were acquired on an upright Leica DMR HC microscope equipped with a color Olympus DP 50 camera using ViewFinder lite dedicated software for acquisition and Olympus Bio System CellF-dedicated software for 2D reconstruction. All images were adjusted for contrast and brightness to equilibrate light levels. Images were cropped, resized, and rotated for presentation's sake. The images were not software modified in any case.

Western blot analysis

In order to quantify OBP-1F protein, we collected the mucus by direct application of sample discs (Wescor, Logan, UT) on undamaged tissues in different regions of the OE: between septum and turbinates and on both external sides of the olfactory turbinates, or inside LNG, or inside the respiratory trachea as a negative control. Anyway, bloody samples were discarded from the study. Humidified sample discs were kept for 30 min in cold buffer (10 mM HEPES-NaOH, 150 mM NaCl, 1 mM EGTA), 1% phenylmethylsulfonyl fluoride, and a cocktail of anti-proteases (Complete, Roche Diagnostics) on a rotating wheel at 4 °C, and then centrifuged at 5000 r.p.m., 10 min. Supernatants were collected and protein levels quantified using BCA protein assay (Pierce, Perbio Science, Brébières, France). To test the presence of OBP-1F, we performed an 8% SDS-PAGE analysis of boiled aliquots (5 min) of 25 µg of total proteins from each extracts. Following electrotransfer, membranes were blocked with 5% no-fat milk at room temperature for 1 h, then incubated overnight at 4 °C in 4.5% no-fat milk containing a custom-made rabbit polyclonal antibody (1:500) developed by Eurogentec (Belgium), and directed against recombinant OBP-1F (Briand et al. 2000). Control using a preabsorbed antibody (1:1 in equimolar concentration) was negative (see Results). After extensive washing in PBS–0.5% milk (3 × 15 min each), the membranes were incubated with goat anti-rabbit secondary antibody coupled with horseradish peroxidase (1:5000, Sigma Aldrich, l'Isle-d'Abeau, France). The targeted proteins were detected using ECL Western Blotting Detection kit (Amersham Biosciences). The integrated density of protein level of each band on the blots was determined by densitometry using the ImageJ software (ImageJ).

Results

Identification of genes differentially expressed following food deprivation

To study the differential expression of genes in the OM of rats following food deprivation, we spotted a nylon membrane with a mixed collection of samples (Table 1). It included

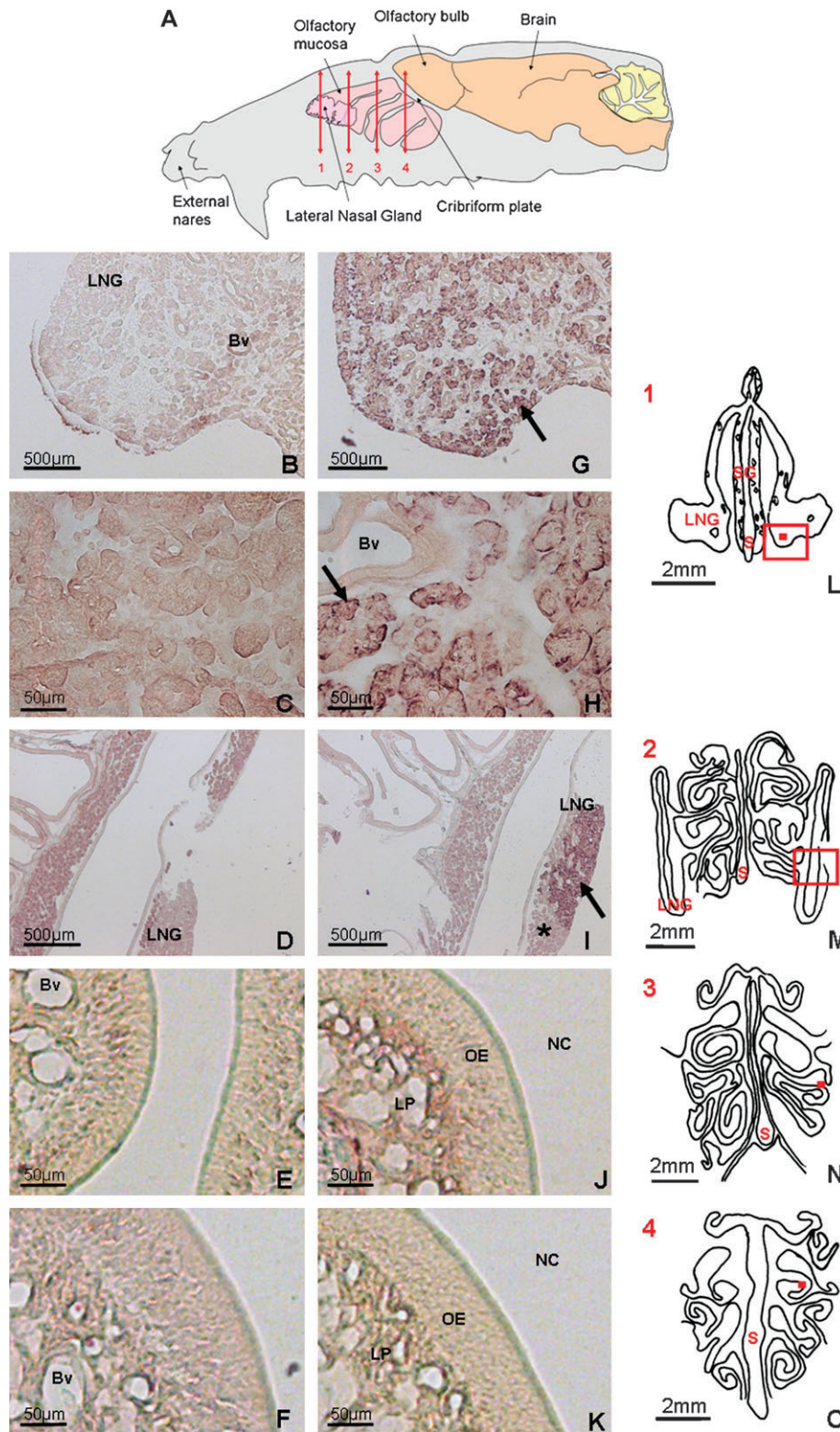


Figure 2 Distribution of OBP-1F mRNA in serial sections of the rat nasal cavity. (A) Schematic diagram of a sagittal section of the rat head showing the position of the LNGs in the nose cavity with respect to other rat head structures. The position of 4 sections studied by ISH is numbered from 1 to 4 by red lines from anterior to posterior region of the head. ISH of OBP-1F mRNA on the 4 serial sections of rat nasal mucosa using antisense RNA probe (G–K) was shown. Control using OBP-1F sense RNA probe (B–F) of consecutive sections did not give any signal. Black arrows indicate labeled structures and asterisk shows acini where no labeling is evidenced (I). Illustrations of the schematic structure of the 4 serial anteroposterior OM sections (L–O). Each red boxes (size depending of the capture scale) shows the position of the structure where the images were captured. Bv: blood vessel; NC: nasal cavity; S: septum; SG: septal gland.

gene fragments ($n = 88$) isolated from a differential display screening between fed and fasted rats. Among them, 77 samples matched with already annotated sequences from the rat and mouse gene index (Table 1), whereas 11 were unidentified in the available databases. A collection of genes involved in neurotransmission ($n = 94$) and some samples of interest ($n = 21$) completed this array (Table 1). Altogether, almost half of the total 203 cDNA spotted on the macroarray ($n = 92$) were linked to a GO term. Those molecules included functions related to cellular and physiological processes, binding, and signal transduction activities (indent 2 of the GO tree; Figure 1). The spotted macroarray was probed with labeled retrotranscribed RNA either from OM of normally fed (fed, $n = 4$) or from 48-h food-deprived rats (fasted, $n = 4$) rats. Normalization and statistical treatment of hybridization signals between fed and fasted rats revealed that 15 molecules were differentially expressed, by at least a 2 fold-factor ($P < 0.05$ in each case): 14 were downregulated in fasted state and only one was up regulated, the ribosomal 18S RNA (Table 2). Out of the 15 sequences, 11 matched known proteins in databases and were classified depending on their biological functions. Four neurotransmitter receptors, but also molecules relevant to the OM function (OBP, OMP, or glial fibrillary acidic protein [GFAP]), were obtained with scores of gene repression ranging from 2.36 to 5.43. Among the unknown molecules, RP23-125 corresponds to a Riken cDNA of mouse, RP23-81P12 is homologous to the human

SEC14L3 gene (tocopherol-associated protein 2) (Kempna et al. 2003), BAC CH230-19C12 corresponds to a repeated sequence already described in rodents, and finally, the 82C sequence has no match in the databases.

Among these 15 candidates, we selected one molecule, the OBP-1F because it displayed the highest gene repression (5.43) and the lowest P -value. Moreover, taking into account that this molecule is mainly produced in the LNG, that is, outside the OM, its expression by the OM was rather unexpected.

OBP-1F transcript is evidenced in LNGs of the rat nasal mucosa by ISH

We explored the tissue distribution of the OBP-1F transcript in the nasal mucosa by ISH of serial sections (1–4) in normally fed 2-month-old Wistar rats (Figure 2A). A schematic representation of each section structure is given in Figure 2L–O. Digoxigenin-labeled antisense probe revealed a strong signal for OBP-1F mRNA, largely confined to LNG structures on sections 1 and 2 in the lateral anterior region of the nasal mucosa (Figure 2G–I), with a clear labeling of acini (arrows) as already described (Pevsner et al. 1988). Some of the other multiple nasal glands were devoid of labeling (data not shown), suggesting an heterogeneity toward the expression of OBP-1F between the different nasal glands (Bojsen-Moller 1964) and between acini composing the

Table 2 List of genes differentially expressed in the OM following food deprivation

Gene ID	Description	Accession number	P -value	Repression ratio fed/fasted
OBP-1F	OBP-1F	NM_138903	<0.001	5.43
RP23-125	RP23-125H15	AL732311	<0.001	4.93
RP23-81P12	RP23-81P12	AL807395	<0.001	4.42
Bac-ch230	BAC CH230-19C12	AC106947	0.001	4.01
LCN 11	Lipocalin 11	XM_575093	0.011	3.20
Grm2	Metabotropic glutamate receptor 2	XM_343470	0.017	2.83
GFAP	Glial fibrillary acidic protein	NM_017009	0.023	2.70
G6PDH	Glucose-6-phosphate dehydrogenase	NM_017006	0.023	2.68
OMP	Olfactory marker protein	RMN26926	0.030	2.60
Chrm2	Cholinergic receptor, muscarinic 2	NM_031016	0.030	2.59
PIP	Prolactin-induced protein	NM_022708	0.030	2.59
82C	nd	—	0.034	2.51
Chrn2	Nicotinic acetylcholine receptor, beta 2 subunit	NM_019297	0.039	2.46
Grm6	Metabotropic glutamate receptor 6	NM_022920	0.049	2.36
18S-RNA	18S-RNA	AF102857	0.045	0.41

Values are fold changes comparing food-deprived rats with normal fed rats. For each sequence, the gene ID, gene name, reference accession numbers on Blast, and function class are specified.

LNG (Figure 2I). In the 2 last anteroposterior sections 3 and 4 (even after long exposure time), no labeling was observed (Figure 2J–K). Negative controls using a sense probe, under identical conditions, showed no staining at all (Figure 2B–F). Therefore, although histological data confirmed transcription of OBP-1F in the LNG as already published (Pevsner et al. 1988), this first survey was unable to disclose the site of OBP-1F expression in the OM.

OBP-1F gene is transcribed both by LNG and OM of rats

In order to clarify the occurrence of OBP-1F mRNA expression in OM itself, we carefully dissected apart each OM and LNG structures from fed adult rats to quantify OBP-1F gene expression levels by qPCR in both tissues (Figure 3A). The relative messenger levels were expressed as a mean normalized to an endogenous reference, β -actin. The OM expressed low, albeit detectable levels of OBP-1F mRNA, with a relative expression level ~ 500 fold less than in the LNG ($n = 5$; $P < 0.05$, Figure 3A). Therefore, a contribution of OM in OBP-1F mRNA transcription inside the nasal mucosa had to be considered in addition to the main site already characterized, that is, the LNG.

A 48-h fasting downregulates OBP-1F mRNA expression in the nasal mucosa

Based on the above data, we undertook a further analysis of OBP-1F mRNA expression to refine the influence of the nutritional status on both structures. Quantitative PCR was performed for OBP-1F gene expression in nasal mucosa isolated either from fed or 48-h fasted rats and then normalized to β -actin gene expression. As depicted in Figure 3B, OBP-1F expression in the OM was decreased almost 3-fold in fasted rats compared with fed rats ($n = 5$ for each; relative

expression: 5.3 ± 1 vs. 1.9 ± 1 , $P < 0.05$). This result is consistent with macroarray data, which showed a more than 5-fold decrease in fasted rats compared with fed ones. A similar quantification of OBP-1F mRNA expression levels in LNG, where mRNA levels are 500-fold more expressed than in OM, revealed no significant difference of mRNA level between fed and fasted rats (Figure 3C). Therefore, only the fraction of OBP-1F mRNA expressed in OM appeared regulated by the nutritional status, suggesting a tissue-specific OBP-1F gene transcription control in response to a 48-h fasting.

OBP-1F protein ratio between LNG and OM is correlated to the mRNA expression ratio

As aforementioned, the differential expression of OBP-1F mRNA between LNG and OM reached a relative expression level of ~ 500 fold. To see whether this ratio was also evidenced at the protein level, we carefully dissected out OM and LNG from fed adult rats and then absorbed the mucus either in contact with the OM or inside the LNG structures. Immunoblot analysis of total protein extracts from these mucus samples ($n = 5$ for each tissue) was performed. Samples blotted were analyzed using anti-OBP-1F antibody and revealed a single specific 19-kDa band corresponding to known OBP-1F apparent molecular mass (Figure 4A). Quantitative analysis showed that OM mucus contains 17-fold less OBP-1F protein when compared with LNG mucus (Figure 4B, $n = 5$). Although the protein ratio between LNG and OM was still correlated to the mRNA expression ratio, the trend is less striking. This decreased ratio can be ascribed to the topology of the nasal mucosa because the extracted mucus in contact with the OM is fully exposed to OBP-1F atomization via the LNG duct. Thus, the OBP-1F contribution from the LNG might lower the differential expression ratio between these 2 tissues. To validate this hypothesis,

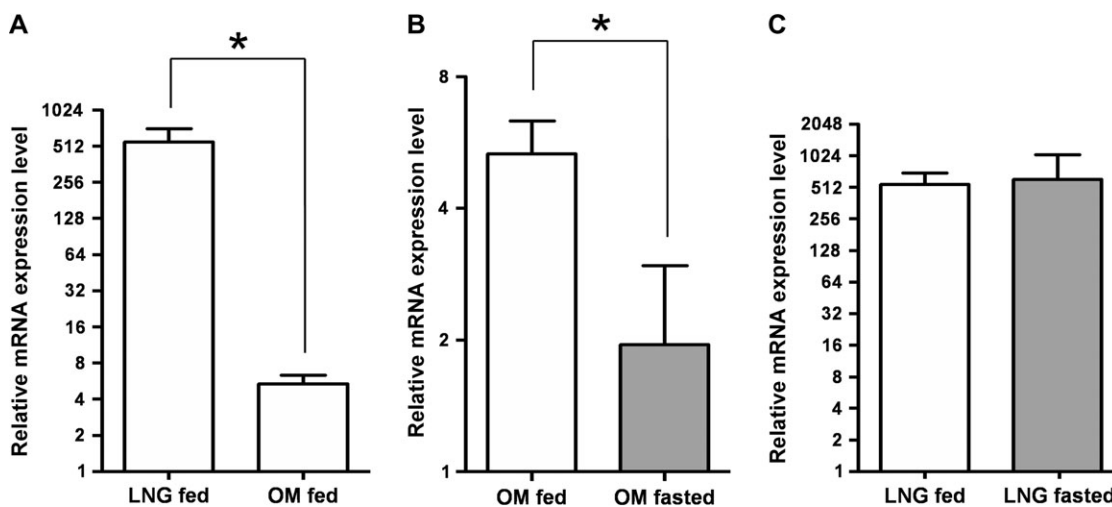


Figure 3 Effect of food deprivation on OBP-1F mRNA level. Relative messenger levels of OBP-1F in OM and LNGs of fed rats (A; $n = 5$ for each group), in OM of fed or fasted rats (B; $n = 5$ in each group) and in LNG of fed ($n = 4$) or fasted ($n = 3$) rats (C). Results are relative to β -actin expression and expressed as mean \pm SEM, significant difference is denoted by * $P < 0.05$ (nonparametric *t* test).

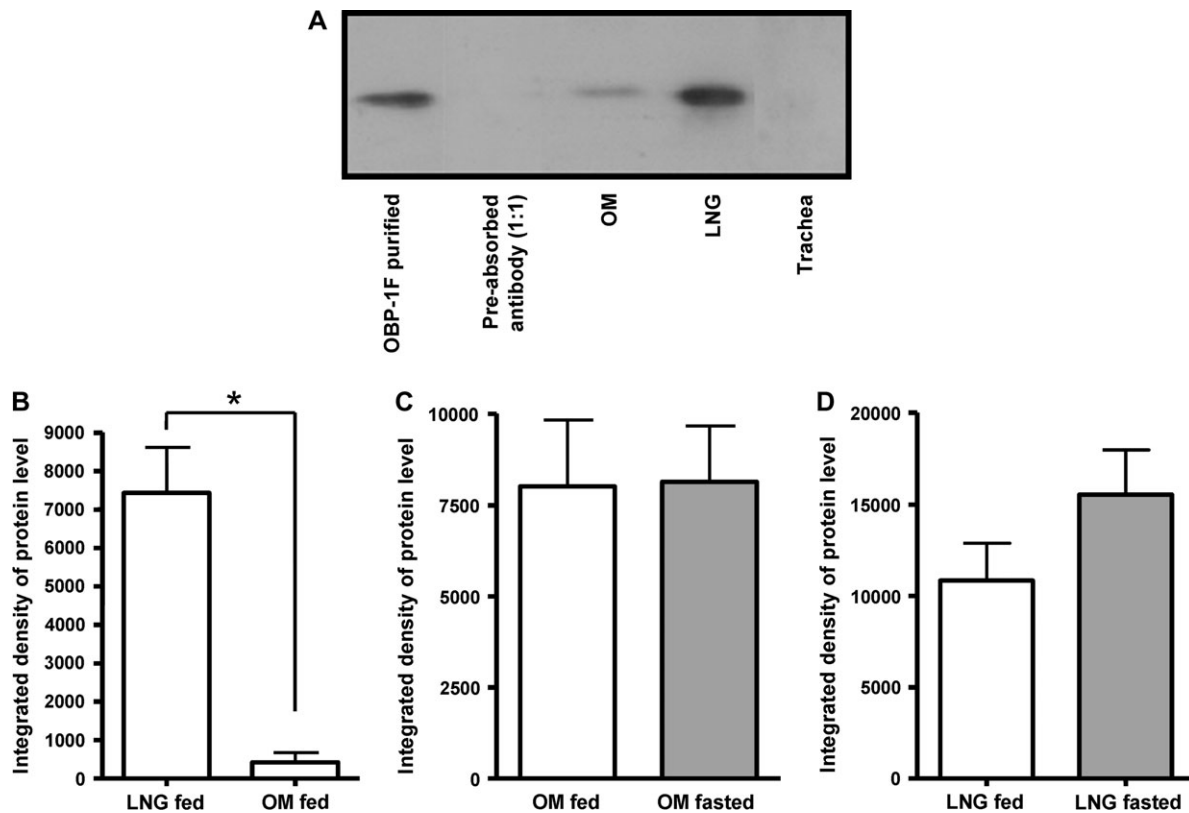


Figure 4 Quantitative analysis of the OBP-1F secretion in both LNG and OM mucus. **(A)** Immunoblotting for OBP-1F in different nasal mucus reveals a single 19-kDa band. Positive control against the purified OBP-1F (lane 1) or negative control after preabsorption of the antibody (lane 2) shows the specificity of the antibody toward OBP-1F. Whereas a single 19-kDa band is revealed in OM (lane 3) as well as in LNG (lane 4), no specific staining is observed in a control mucus extracted from trachea (lane 5). **(B)** Comparison of OBP-1F expression level in OM ($n = 5$) and LNGs ($n = 6$) of fed rats. **(C and D)** Effect of food deprivation on OBP-1F protein level in OM (C; $n = 5$ in each group) and LNGs (D; $n = 5$ in each group). The integrated density of each band on the blots was determined by densitometry using the ImageJ software. Results are expressed as mean \pm SEM; significant difference is denoted by $*P < 0.05$ (nonparametric t test).

a differential expression study of OBP-1F between fed and fasted rats was undertaken by immunoblot analysis of mucus either in contact with LNG (Figure 4C) or OM (Figure 4D). As expected, this study revealed no significant difference between the 2 experimental conditions either at the LNG or OM level.

Discussion

We demonstrated here that starvation is followed by transcriptional changes in the OM. We identified several genes whose expression was regulated by the nutritional status in the OM of rats.

The combination of our differential display analysis together with the screening of a macroarray was well adapted to study this situation where few samples had to be analyzed. After ddmRNA, we found a relatively low number of unknown genes (11) following annotation on the rat, mouse, or human genomes. They might have resulted from cDNA sequence modifications during the whole complex molecular biology procedure, resulting in some limitations in the use of ddmRNA alone. In addition, the macroarray screening iden-

tified only 7% of potential candidates of 203 molecules selected by ddmRNA, confirming that the latter approach generates a high number of false-positive results and requires additional screening (Matz and Lukyanov 1998). The 2-fold change criteria used for transcriptomic studies together with our highly stringent statistical analysis rejected certainly candidates from the ddmRNA screening; however, this enables us to affirm that selected candidates were highly relevant.

It cannot be ruled out that part of the changes might result from the enhanced oxidative stress with fasting as previously described (Sorensen et al. 2006). However, none of the molecular systems known to be involved in stress response have been highlighted during the ddmRNA screening.

Due to the structure of the nasal tissue, a small amount of intermingled respiratory mucosa partly participates in the OM sampling during the extraction procedure, possibly leading to a biased analysis. However, results of the macroarray assay indicate that the samples are homogenous in terms of ratio between olfactory and respiratory mucosae. Indeed, some of the screened molecules, such as one olfactory receptor (NM_001000932) or the neuronal nitric oxide synthase, Nos1 (NM_052799), are neuron specific and do not

display any change of their expression level (Broillet and Firestein 1996). In addition, among the 94 molecules involved in neurotransmission, 53 of them exhibiting a median spot intensity 2-fold up to the median background keep a constant expression level between nutritional conditions (not shown).

Among the 15 sequences selected as being regulated by the nutritional status, some belong to olfactory or neuronal tissue-specific class, such as the olfactory marker protein or one OBP, whereas other are more related to metabolic cues (18S RNA or glucose-6-phosphate dehydrogenase), which was rather expected (Wensley et al. 1995). Other sequences, such as RP23-81P12, a rat sequence homologous to the human gene SEC14L3 (tocopherol-associated protein 2, TAP2), which encodes a transcription factor depending on tocopherol (vitamin E) (Kempna et al. 2003), might be considered. Actually, a vitamin E deprivation is depicted as one of the diseases that cause olfactory dysfunction (Henkin and Hoetker 2003). Thus, TAP2 might play a role in olfactory modulation through its downregulation following food deprivation.

Some candidates belonging to the neurotransmitter receptors family also exhibit a downregulation in OM of fasted rats: 2 metabotropic glutamate receptors (Grm2, Grm6) and 2 cholinergic receptors, one nicotinic (Chrn2) and one muscarinic (Chrm2). The cholinergic receptor subtypes were previously identified in the OM of amphibian and fish (Getchell ML and Getchell TV 1992; Drescher et al. 2004), whereas the G-protein-coupled metabotropic glutamate receptors were only described at the OB level (Sahara et al. 2001). A potential target might be the olfactory ensheathing cells (OECs) located in the LP that wrap olfactory neuron axons in the olfactory nerve. Those metabotropic glutamate receptors have been suggested to modulate synaptic transmission via both pre- and postsynaptic effects (Sahara et al. 2001). Interestingly, 3 of our candidates (Grm2, Grm6, and Chrm2) are coupled to the α Gi protein, which results in the inhibition of adenylyl cyclase (Pin and Duvoisin 1995; Fryer and Jacoby 1998). Thus, according to the nutritional status, Grm2 and Grm6 might modulate the olfactory signal transmission toward the OB, by acting at the presynaptic level of the synaptic junction between olfactory neurons and mitral cells. Indeed, metabotropic receptors subtypes expressed by glial cells participate in the communication with neurons in the developing OB through calcium signaling (Rieger et al. 2006). However, only the cellular distribution of those receptors at the light and electron microscopic level would help to clarify their potential roles in olfactory neuromodulation.

In our study, we show that the GFAP mRNA, which is expressed by OECs of the LP, is downregulated by the nutritional status, possibly by changes in circulating hormonal levels. Indeed, GFAP was already shown as being modulated by cytokines, steroid hormones, or growth factors (Laping et al. 1994). More interestingly, Dennis et al. (2005) reported

that GFAP was downregulated in OECs extracted from OM of type-1 diabetic rats, thus linking a diminution of insulin receptor signaling to glial cell function changes. The resulting changes of GFAP might also disturb the neuron–glia interactions, as suggested above for the metabotropic receptors.

More interestingly, we highlighted one OBP, OBP-1F, as displaying the 2 characteristics of 1) being physiologically relevant to olfaction and 2) presenting the highest repression upon food restriction.

Our macroarray, western blot, and q-PCR data pointed out the OM as an OBP-1F-secreting tissue, in addition to its main site, that is, the LNGs (Pevsner et al. 1988). This contribution seems to be minor because OBP-1F mRNA expression is about 500-fold less expressed in OM than in the LNG's acini. This may result either from the presence of discrete synthesis sites spread all over the OM or from numerous cells transcribing low levels of OBP-1F gene. If nasal glands were described to be the unique site of synthesis for 2 OBP-related proteins in mouse (Pes et al. 1998), few data showed an expression of OBP by Bowman's glands in bovine or *Xenopus* species (Pevsner et al. 1986; Millery et al. 2005). However, our present study failed to detect any labeling of those secreting glands either by ISH or ICC in the rat OM.

More interestingly, our work indicated that OBP-1F gene expression was downregulated by a 48-h food deprivation only in the OM, that is, in a tissue-specific way. Despite their close anatomical localization, each secretory structure was differentially affected by the nutritional status in terms of the transcriptional control of OBP-1F gene expression. Recently, a microarray-based study of OBP genes expression in the mosquito showed that whereas some OBPs were overexpressed in the female after a blood feeding, most of them were downregulated (Biessmann et al. 2005). In addition, olfactometer assays demonstrated that host seeking was drastically inhibited after a blood meal in the *Anopheles gambiae* (Takken et al. 2001). However, none of the 2 other known OBP in the mucus of the rat species was highlighted by our ddmRNA analysis. Contrary to the insect where interactions between odorant ligands and members of the large family of OBPs are quite specific, the lack of specificity between a mammalian OBP and an odorant class prevents further hypotheses on having selected OBP-1F only.

Among several factors that might fluctuate following food deprivation (cytokines, growth factors) and might influence OBP-1F transcription, we should also point out leptin, a well-known circulating cytokine. Convincing facts, such as 1) leptin expression is regulated by the nutritional status in the rat OM (Baly et al. 2007), 2) leptin receptors are expressed by Bowman's glands of the OM, 3) ob/ob leptin-deficient mice display olfactory dysfunctions (Getchell et al. 2006), and 4) a perfusion with leptin induces an increase of the intestinal mucus secretion in rats (Plaisancie et al. 2006), argue that leptin might be a candidate to the olfactory

modulation by the nutritional status through the control of mucus composition.

At the protein level, the mucus in contact with the OM displays 17-fold less OBP-1F protein when compared with LNG mucus, thus confirming to a less extent the variation of mRNA expression ratio. In addition to several post-transcriptional regulations that might extensively modify this protein:mRNA ratio, a simple hypothesis could be added. Taking into account the anatomical structure of the rat nose, atomization of OBP-1F from the LNG duct caused by the sniffing largely contributes to the composition of the OM mucus (Pevsner et al. 1988), thus lowering the differential expression ratio between these 2 parts of the nasal secretions. Obviously, knowing the absence of nutritional regulation of OBP-1F mRNA in the LNG, macroscopic effects on OBP-1F at the OM level could not be evidenced. However, we could speculate that the fine transcriptional control of OBP-1F in the OM, as demonstrated above, display functional consequences at the local level in relation to one (or more) of the several functions of these molecules in early olfactory perireception events: 1) reception of the odorant molecules upon their arrival inside the nasal cavity (Pelosi 1994), 2) elimination of odorous molecules in excess to prevent the saturation of the olfactory receptors (Schofield 1988; Burchell 1991), and 3) clearance of toxic substances to protect the OM against injuries (Boudjelal et al. 1996). According to our results, the OBP-1F secreted by LNG might play a role in defense mechanism by countering possible toxicity, whereas the OBP-1F produced by the OM itself might also act locally to regulate the mucus composition for the control of *in vivo* odorant responsiveness (Oka et al. 2006). Knowing the broad spectrum of activity found for mammalian OBP in the olfactory mucus and their high turnover rate (Pelosi 2001), a local modification of the mucus composition might rapidly affect its physicochemical or biological properties, possibly through the dynamics of dimerization of OBP-1F (Nespoulous et al. 2004). Similarly, a cytochrome P450 overexpression in the rat nasal mucosa following a 72-h fasting was proposed to improve clearance of hormones, drugs, or inhaled odorants (Longo et al. 2000).

Among the 2 aforementioned expression sites for OBP-1F mRNA, only the OM fraction is under a fine transcriptional control following food deprivation. It could regulate the local molecular environment close to its site of secretion to participate in the perireceptor events of the olfactory signal reception. In conclusion, these data are also in agreement with a possible involvement of the nutritional status in OM functions through the indirect control of secretory functions of the OM.

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