Induction of NPY/AgRP Orexigenic Peptide Expression in Rat Hypothalamus is an early Event in Fasting: Relationship with Circulating Leptin, Insulin and Glucose

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Key Words

Short-term fasting • NPY • AgRP • POMC • CART • Leptin • Ghrelin • Insulin • Glucose • Hypothalamic feeding control

Abstract

Hypothalamus is crucial in the control of energy intake and expenditure in mammals, presenting two interconnected populations of neurons producing orexigenic NPY/AgRP (neuropeptide Y; agouti related peptide) and anorexigenic POMC/CART (pro-opiomelanocortin; cocaine and amphetamine regulated transcript) neuropeptides. We aimed to shed more light on the response and sensitivity in the production of these neuropeptides to face nutritional changes, particularly food deprivation, and on the signals that regulate them. Male Wistar rats were fasted for 0, 4, 8 and 24h and refed for 3h after 8h fasting. mRNA levels of gastric and adipose tissue (retroperitoneal, mesenteric and inguinal) leptin, and of hypothalamic NPY, AgRP, POMC, CART, leptin receptor, SOCS3 (suppressor of cytokine signaling 3) and insulin receptor were analyzed. Gastric and circulating leptin, and circulating insulin, glucose and ghrelin were also determined. The only neuropeptide mRNAs that responded (increasing) to the short-term

periods of fasting used were those of NPY (transiently) and AgRP, and these changes were accompanied by an increase in leptin receptor mRNA levels and by a decrease in adipose and gastric leptin expression and in the circulating levels of leptin, insulin and glucose, but without changes in circulating ghrelin. The elevation in AgRP and leptin receptor mRNA levels and the drop in circulating leptin were not reverted with refeeding. It is suggested that the induction of expression of the orexigenic molecules in NPY/AgRP neurons is an early event upon fasting, related with changes in leptin, insulin and glucose levels, but with the role of leptin signaling in particular.

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Introduction

Satiety and appetite are feelings regulated in mammals by a complex system of peripheral and central signals, which modulate food intake and energy balance. In the central nervous system (CNS), the arcuate nucleus of the hypothalamus is crucial for feeding control and contains two interconnected groups of “first order” neurons producing neuropeptide Y (NPY) and agouti related peptide (AgRP), and pro-opiomelanocortin

(POMC) and cocaine and amphetamine regulated transcript peptide (CART) [1]. These are the most important neuronal populations that regulate energy homeostasis in the hypothalamus, serve as primary sensors for circulating adiposity signals, and project axons to “second order” neurons, generating a cascade response [2, 3]. NPY is orexigenic and has been described as the most powerful central enhancer of appetite, and it is also expressed in other hypothalamic areas apart from the arcuate; in the arcuate, its expression is abundantly co-localized with AgRP expression, which is also a potent orexigenic, but synthesized exclusively in the arcuate [1, 4]. AgRP influences food intake mainly through the competitive antagonism of central melanocortin receptors (see below) and, alternatively, maybe by mediation of orexin or opioid receptors [1]. POMC is the precursor of several molecules and, importantly, of the anorexigenic alpha melanocyte stimulating hormone (α -MSH), which is the main regulator of energy balance in the melanocortin family and mediates its anorexigenic effects through melanocortin receptors [1]. CART is the other anorexigenic important peptide and, similarly to NPY/AgRP neurons, 90% of CART neurons are co-localized with POMC neurons in the arcuate nucleus [1]. In the complex system of hypothalamic signals regulating energy homeostasis, there is an interconnection between NPY/AgRP and POMC/CART neurons in the arcuate, where the NPY/AgRP neurons can regulate POMC neurons by an inhibitory input [5]. These hypothalamic circuits regulate energy balance both by control of food intake and energy utilization, and they have effects on secretion of fat metabolism regulating hormones [2]. In return, hormones from fat stores and other tissues, as well as other peripheral circulating signals, can regulate the response of NPY/AgRP and POMC/CART neurons. Among the hormonal signals, leptin, insulin and ghrelin stand out.

Leptin is mainly secreted by the adipose tissue, in proportion to fat stores, and can enter the CNS, where crucial leptin receptors are found, notably in the arcuate nucleus [6]. Leptin has potent effects on feeding behaviour, thermogenesis and neuroendocrine responses, provoking a decrease in food intake and an increase in energy expenditure [7, 8]. Adipose derived leptin has been related with the long term control of energy homeostasis and has also been suggested as a primary starvation signal (when its levels rapidly fall) [8, 9]. It is also produced by the stomach [10-12], and this gastric leptin has been involved in the short-term regulation of food intake [8]. Both NPY/AgRP and POMC/CART arcuate neurons

express the long form of the leptin receptor (OB-Rb) (a class I cytokine receptor, crucial for the mediation of leptin effects) [3, 9, 13], although the leptin receptor is also expressed in other regions of the hypothalamus and not only in the arcuate nucleus [14]. Besides, it must be noted that the effect of leptin can be different depending on the hypothalamic nuclei affected; for instance, in the obese state, NPY/AgRP and POMC neurons in the arcuate can show specific resistance to leptin while other hypothalamic regions keep their sensitivity [15, 16]. Leptin helps to terminate its own action, importantly by induction of the suppressor of cytokine signaling 3 (SOCS3) [17], which might also be involved in the attenuation of the insulin receptor signaling [18].

It seems clear that leptin signaling is a specialized energy balance system that emerged during vertebrate evolution, but recent works suggest that also the more ancient and common metazoan insulin (or insulin-like, depending on the species) signaling could also have an important role in the brain for the regulation of energy balance in mammals, partially overlapping with the leptin system [19, 20]. In mammals, insulin, like leptin, is secreted in proportion to fat stores, and also enters the CNS in proportion to its plasma levels [21]. Insulin receptors are expressed in neurons of brain areas involved in feeding control, and central administration of insulin can reduce food intake and body weight; moreover, insulin, like leptin, has been shown to affect neuropeptide expression in the hypothalamus [4, 21]. Insulin transmits its signal through the insulin receptor (InsR), a membrane-bound tyrosine kinase receptor, particularly highly expressed in the arcuate nucleus and found both in POMC and AgRP neurons [21]. In general, NPY/AgRP neurons are directly inhibited by both insulin and leptin, while POMC/CART neurons are stimulated by these hormones [3, 19].

Ghrelin is the primary orexigenic signal from the periphery and has been reported to exert its orexigenic effects through circulation [22] and by suppressing gastric vagal afferents [23]. Ghrelin is mainly produced by the stomach [24] and can increase food intake and body weight in both rodents and humans, with a suggested role in energy balance [25-28]. Moreover, ghrelin is sensitive to the nutritional state, since circulating ghrelin levels have been found to increase by fasting and decrease after refeeding in several species [27, 29-33], and the refeeding-related decrease is dependent on the type of macronutrient (higher for carbohydrates) [34]. The ghrelin orexigenic effects appear to be mediated, at least in part, by activation of NPY/AgRP neurons (where ghrelin receptor is expressed) [13]; in fact, ghrelin administration

Table 1. Nucleotide sequences of primers and cycling conditions used for PCR amplification. Abbreviations: NPY, neuropeptide Y; AgRP, agouti related peptide; POMC, pro-opiomelanocortin; CART, cocaine and amphetamine-regulated transcript; OB-Rb, long isoform of the leptin receptor; SOCS3, suppressor of cytokine signaling 3; InsR, insulin receptor; 18S, ribosomal RNA 18S (reference gene).

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Cycling Conditions	Amplicon Size (bp)
Leptin	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	95°C for 2s 58°C for 6s 72°C for 10s	186
NPY	TGGACTGACCCTCGCTCTAT	GTGTCTCAGGGCTGGATCTC	95°C for 2s 62°C for 6s 72°C for 12s	188
AgRP	AGAGTTCTCAGGTCTAAGTCT	CTTGAAGAAGCGGCAGTAGCACGT	95°C for 2s 59°C for 6s 72°C for 16s	210
POMC	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGT TC	95°C for 2s 62°C for 6s 72°C for 12s	266
CART	AGAAGAAGTACGGCCAAGTCC	CACACAGCTTCCCGATCC	95°C for 2s 55°C for 6s 72°C for 6s	84
OB-Rb	TGAAACATTGAGCATCTTT	CGATGCACTGGCTGACAGAA	95°C for 2s 58°C for 6s 72°C for 16s	368
SOCS3	TTCGGGACTAGGTAGGAAGGA	AGGGCCCCAGTCTGAGTATT	95°C for 2s 56°C for 6s 72°C for 8s	123
InsR	CTCCTGGGATTTCATGCTGTT	GTCCGGCGTTCATCAGAG	95°C for 2s 60°C for 6s 72°C for 10s	242
18S	CGCGGTTCTATTTTGTGGT	AGTCGGCATCGTTTATGGTC	95°C for 2s 60°C for 6s 72°C for 10s	219

increases NPY expression and a competitive interaction between ghrelin and leptin in feeding regulation has been suggested [25].

Apart from hormonal signals, metabolic signals are also important for the brain. In this sense, glucose levels are also involved in the regulation of the activity of neurons that regulate appetite [35]. For instance, food deprivation and the concomitant decrease in blood glucose lead to an increase in the expression of both NPY and AgRP and a reduction of POMC and CART expression [36]. Moreover, hormonal (leptin and insulin) and metabolic (glucose levels) signals can converge in an integrated way to regulate the expression of orexigenic/anorexigenic neuropeptides [3, 36, 37].

In an important number of the above mentioned studies, the use of the fasting model has been crucial to shed light into the possible regulators and mechanisms related to the control of food intake and energy balance in the hypothalamus. Nevertheless, when using animal models such as rodents, relatively long times of fasting (from 24 h onwards) have been used in most studies. We aimed to gain further insight into the hypothalamic orexigenic/anorexigenic neuropeptide expression regulation by the above mentioned signals and we studied in rats the chronological changes with fasting (starting from the very early stages) and refeeding in leptin production (both of gastric and adipose origin and studying the differences of leptin production between distinct fat depots), in insulin, ghrelin and glucose levels, in the hypothalamic mRNA expression levels of the orexigenic/

anorexigenic neuropeptides NPY, AgRP, POMC and CART, and in the hypothalamic mRNA expression of the molecules related to the signaling pathways of the hormones whose circulating levels suffered changes, i.e. OB-Rb, SOCS3 and InsR. This model has allowed us to decipher the earliest responders among these signals and genes and to uncover transitory changes that can be of importance in later responses shown during fasting.

Materials and Methods

Animals and sample collection

Three-month-old male Wistar Rats (Charles River Laboratories España, SA, Barcelona, Spain), housed at 22°C with a period of light/dark of 12 h (lights on from 08:00 to 20:00) and fed with a standard chow diet (Panlab, Barcelona, Spain) with free access to food and water, were randomly distributed under 5 different feeding conditions (n= 6-8): *ad libitum* feeding state, 4, 8 or 24 h fasting and 3 h refeeding after 8 h fasting. The fasting period started at the beginning of the dark phase in the 4 and 8 h fasting groups and at the beginning of the light phase in the 24 h fasting group. *Ad libitum* animals were sacrificed at the beginning of the light phase. To design the fasting protocol, we considered that rats eat most of their daily food intake at night [38], thus the 4 and 8 h food deprivation periods were during the dark cycle, and just before the beginning of the light cycle.

After killing the animals by decapitation, the retroperitoneal, mesenteric and inguinal white adipose tissue (WAT) depots and the whole hypothalamus were rapidly removed, frozen in liquid nitrogen and stored at -70°C until RNA analysis. The hypothalamus was harvested by using the

	<i>Ad libitum</i>	4 h fasting	8 h fasting	24 h fasting	8 h fasting + 3 h refeeding
Body weight before fasting (g)		366 ± 7	368 ± 2	367 ± 4	366 ± 3
Body weight at sacrifice (g)	366 ± 3 (a)	359 ± 7 (a,b) †	349 ± 3 (b) †	335 ± 4 (c) †	358 ± 3 *
Gastric contents (g)	6.24 ± 0.49 (a)	1.83 ± 0.29 (b)	0.87 ± 0.42 (b,c)	0.63 ± 0.14 (c)	7.36 ± 0.60 *
Glucose (mg/dl)	104 ± 3 (a)	98 ± 2 (a)	88 ± 3 (b)	79 ± 3 (c)	109 ± 3 *
Insulin (µg/l)	1.30 ± 0.15 (a)	0.637 ± 0.066 (b)	0.479 ± 0.083 (b,c)	0.340 ± 0.019 (c)	1.03 ± 0.18 *
Leptin (µg/l)	2.20 ± 0.30 (a)	1.20 ± 0.21 (b)	0.803 ± 0.135 (b,c)	0.428 ± 0.076 (c)	1.17 ± 0.32 §
Ghrelin (µg/l)	2.10 ± 0.39	2.44 ± 0.70	2.84 ± 0.50	2.80 ± 0.63	2.95 ± 0.51

Table 2. Body weight, weight of gastric contents and blood parameters. Body weight before fasting and at sacrifice, weight of gastric contents, and circulating levels of glucose, insulin, leptin and ghrelin in rats under different feeding conditions: ad libitum feeding state, after 4, 8 or 24 h fasting, and after 3 h refeeding following 8 h fasting. Data are means ± SEM (n = 6 to 8). a≠b≠c (p<0.05, one-way ANOVA and LSD post-hoc test). † body weight at sacrifice vs. body weight before fasting (p<0.05, repeated measures Student's t test). * 3 h refeeding after 8 h fasting vs. 8 h fasting (p<0.05, Student's t test); § 3 h refeeding after 8 h fasting vs. ad libitum (p<0.05, Student's t test).

following landmarks, i.e., frontal edge of the optical chiasm, lateral sulci, caudal edge of the mammary bodies, and a depth of 2 mm, as previously described [39]. The gastric contents were weighed, the stomach was opened and rinsed with saline containing 0.1% DEPC (diethyl pyrocarbonate) (Sigma, Madrid, Spain) and the epithelium was scraped off using a glass slide, then it was also frozen in liquid nitrogen and stored at -70°C. Blood was also collected in a heparinized glass and kept at 4°C (for less than 1 h), until it was centrifuged at 700 g for 10 minutes to collect the plasma.

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University and guidelines for the use and care of laboratory animals of our University were followed.

RNA isolation

Total RNA was extracted from tissues by Tripure Reagent (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nadrop Technologies Wilmington, Delaware USA) and its integrity confirmed using agarose gel electrophoresis.

RT-qPCR analysis

Real-time polymerase chain reaction was used to measure mRNA expression levels of leptin mRNA in the stomach and in the white adipose tissue and NPY, AgRP, POMC, CART, OB-Rb, SOCS3 and InsR in the hypothalamus.

Total RNA was reverse transcribed to cDNA as previously described [40]. Real-time PCR was completed using the LightCycler System with SYBR Green I sequence nonspecific detection (Roche Diagnostic GmbH, Mannheim, Germany). Each PCR was performed as previously described [40]. The primers and cycling conditions used are described in Table 1, and all primers were obtained from Sigma (Madrid, Spain).

Quantification of plasma and gastric mucosa leptin concentration

Leptin concentration was determined in the gastric mucosa and in plasma. Gastric mucosa samples were homogenized at 4°C in 1:3 (w:v) of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4) in a Teflon/glass homogenizer. The homogenate was centrifuged at 7000 g for 2 min at 4°C and the supernatant was used for leptin quantification.

Leptin concentration in the gastric mucosa homogenates and in plasma was measured with a mouse leptin enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Min, USA).

Quantification of plasma insulin, ghrelin and blood glucose levels

Plasma insulin concentration was measured using an ELISA kit (DRG Instruments GmbH, Marburg, Germany). Plasma ghrelin concentration was measured with a rat ghrelin enzyme immunosorbent assay kit (Phoenix Europe GmbH, Karlsruhe, Germany) which detects total ghrelin (no only the acylated active form). Blood glucose concentration was measured by an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain).

Statistical Analysis

All data are expressed as the mean ± SEM. Differences between groups were assessed by one-way ANOVA followed by least significances difference post-hoc comparison to assess statistical differences between the groups. Student's *t*-test was used to assess single differences of 8 h fasting with the refed or with the control group. A repeated measures *t*-test was performed to analyze the time-course body weight data. A simple correlation analysis of the studied parameters was also carried out and the Pearson's correlation index is given. The

Fig. 1. Chronological changes in the expression of leptin mRNA in different adipose tissue depots, under fasting and refeeding. (A) Sequential analysis upon fasting: control *ad libitum* feeding state (Ad Lib), after 4, 8 or 24 h fasting (4h F, 8h F and 24h F). (B) 3 h refeeding after 8 h fasting (8h F + 3h refeed) vs. 8h F and control (broken line). mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of animals under *ad libitum* feeding conditions. Data are means $\pm$ SEM (N=6 to 8). One-way ANOVA was performed for each depot, followed by LSD post hoc test: bars not sharing a common letter are significantly different ($p < 0.05$). A Student's t-test analysis was used to determine, for each depot, statistical differences of the refeed group vs. 8h fasted (*) and control *ad libitum* (§) group ($p < 0.05$). WAT, white adipose tissue; r, retroperitoneal; m, mesenteric; i, inguinal.

analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at $p < 0.05$.

Results

Sequential changes in body weight, gastric contents and blood glucose, insulin, leptin and ghrelin levels during fasting

The changes in the weight of the animals, the gastric contents and the circulating parameters analyzed are shown in Table 2. As expected and described before [41], there was a gradual decrease in body weight, gastric contents, circulating glucose and circulating insulin, which were restored to control levels upon 3 h refeeding (after 8 h fasting). Moreover, leptin followed a parallel decrease in blood, which was already significant after 4 h fasting (as for insulin) and continued decreasing with the time-course of fasting, reaching the lowest levels at the 24 h fasting time point, and increasing after 3 h refeeding (after 8 h fasting), although, differently to insulin, it did not reach values comparable to the control ones. On the other hand, there were no significant changes in circulating ghrelin levels either upon fasting or refeeding.

Sequential changes upon fasting and effect of refeeding on leptin mRNA levels in retroperitoneal, mesenteric and inguinal WAT depots

As shown in Figure 1A, leptin mRNA levels decreased in WAT during fasting in all the depots studied, and the decrease was already found after 4 h fasting. These levels were even lower after 8 and 24 h fasting in the retroperitoneal and mesenteric ones. Upon 3 h refeeding after 8 h fasting, the leptin mRNA levels were restored in the iWAT, partially restored in the rWAT and not restored at all in the mWAT (Figure 1B).

Sequential changes upon fasting and effect of refeeding on gastric leptin mRNA and protein levels

Leptin mRNA levels in the gastric mucosa (Figure 2A) were significantly decreased after 4 h fasting and remained low after 8 h fasting, but were slightly raised at the 24 h fasting time point (which levels were not significantly different to the control ones). Leptin levels in the mucosa were also lowered by the fasting condition, although with a delayed pattern compared to the mRNA levels, becoming significantly lower than control ones only after 24 h fasting. 3 h refeeding after 8 h fasting caused a rise in leptin mRNA levels as compared to the 8 h fasting levels, and were not different to the control levels, whereas gastric leptin levels remained low upon 3 h refeeding and were even significantly lower than the control levels (Figure 2B).

Sequential changes upon fasting and effect of refeeding on hypothalamic mRNAs related to the control of food intake and the signaling of leptin and insulin

Figure 3 shows the changes upon fasting and refeeding in the expression of NPY, AgRP, POMC, CART, OB-Rb, SOCS3 and InsR at the mRNA level, in

Fig. 2. Chronological changes in the expression (mRNA and protein levels) of leptin in the stomach, under fasting and refeeding. (A) Sequential analysis upon fasting: control *ad libitum* feeding state (Ad Lib), after 4, 8 or 24 h fasting (4h F, 8h F and 24h F). (B) 3 h refeeding after 8 h fasting (8h F + 3h refed) vs. 8h F and control (broken line). mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of animals under *ad libitum* feeding conditions. Leptin concentration in the gastric mucosa was measured with a mouse leptin enzyme-linked immunosorbent assay and is expressed as pg of leptin per mg of mucosa. Data are means $\pm$ SEM (N=6 to 8). One-way ANOVA was performed for both mRNA and protein results, followed by LSD post hoc test: bars not sharing a common letter are significantly different ($p < 0.05$). A Student's t-test analysis was used to determine, for each depot, statistical differences of the refed group vs. 8h fasted (*) and control *ad libitum* (§) group ($p < 0.05$).

the hypothalamus of rats. With regard to the feeding control neuropeptide genes, only the orexigenic ones (NPY and AgRP) suffered a change in their expression, by raising their mRNA levels, whereas the anorexigenic neuropeptide genes (POMC and CART) did not alter their expression at the mRNA level (Figure 3A). The elevation of mRNA levels was transitory for NPY, becoming significantly higher than control ones after 4 h fasting and decreasing afterwards to levels not significantly different to those of controls. The elevation of AgRP

Fig. 3. Chronological changes in the mRNA expression of hypothalamic genes related to orexigenic and anorexigenic neuropeptides, and leptin and insulin signaling, under fasting and refeeding. (A) Sequential analysis upon fasting: control *ad libitum* feeding state (Ad Lib), after 4, 8 or 24 h fasting (4h F, 8h F and 24h F). (B) 3 h refeeding after 8 h fasting (8h F + 3h refed) vs. 8h F and control (broken line). mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of animals under *ad libitum* feeding conditions. Data are means $\pm$ SEM (N=6 to 8). One-way ANOVA was performed for each type of messenger, followed by LSD post hoc test: bars not sharing a common letter are significantly different ($p < 0.05$). A Student's t-test analysis was used to determine, for each messenger, statistical differences of the refed group vs. 8h fasted and control *ad libitum* (§) group ($p < 0.05$).

mRNA expression was higher and also already significantly different after 4 h fasting and remained elevated at the 8 and 24 h fasting time points.

With respect to OB-Rb mRNA expression, it rose significantly after 8 h fasting and remained elevated after 24 h fasting, while SOCS3 and InsR mRNA levels were not significantly altered by fasting.

3 h refeeding after 8 h fasting did not revert the rise in AgRP and OB-Rb mRNA levels, which remained elevated (Figure 3B). The other mRNAs studied did not

show statistical differences when comparing the refed with either the 8h fasted or the control group.

Discussion

Food deprivation implies an important challenge for the organism and different tissues must respond rapidly and coordinately to face the negative energy balance [41]; among them, the response of the hypothalamus, especially by the NPY/AgRP and POMC/CART producing neurons, is of outstanding importance. In this sense, we report here that the production of these neuropeptides is regulated differently and that only the increase in the orexigenic ones, i.e. NPY and AgRP, happens at the very beginning of the fasting condition, in accordance with changes in the circulating levels of leptin, insulin and glucose levels.

By describing the chronological changes with fasting in our model, it is observed that the expected fall in the circulating levels of leptin, insulin and glucose is gradual and that this fall is already significant for the hormones (leptin and insulin) earlier (after 4 h fasting) than for the glucose levels (significantly decreased at the 8 h time point). On the other hand, the absence of significant changes in circulating ghrelin levels (from the beginning of fasting until 24h) suggests that the changes observed here in the expression of the hypothalamic genes analyzed would probably be more dependent on factors such as leptin, insulin and glucose levels (and maybe others) rather than on ghrelin action. Nevertheless, Ghrelin has been proposed to have a main role as an initiator of meals in the normal daily feeding rhythms [30, 38], and in some studies circulating ghrelin levels have been shown to rise in rats after fasting periods longer than the ones used here, such as a 48 h [27] and 72 h fasting [42] but also, in other studies, already after 24 h fasting in the gastric vein (but not in the truncal vein with 48 h fasting) [31], or in the overall circulation in lean but not in obese rats [43]. Thus, although the present results suggest that short-periods of fasting are not enough to change its overall circulating levels, the discrepancy of the 24 h result with the rise reported by other authors at this same period points out that the influence of experimental circumstances such as the strain and age of the animals, as well as and the method used for measuring ghrelin levels, cannot be discarded.

Since adipose tissue derived leptin is an important indicator of the state of the fat stores and has been described as a primary starvation signal [9], the results obtained here support this idea and show how the response

in leptin production happens very quickly after the beginning of fasting, with a decrease in leptin mRNA expression in all the adipose tissue depots studied, already significant after 4 h fasting (independently of their subcutaneous or internal origin). This decrease in expression is reflected in a decrease in circulating leptin levels, also significant after 4 h fasting, even before the significant drop in blood glucose. Gastric leptin production follows a similar pattern at the mRNA level, with a rapid, and even more marked, fall upon fasting (although with a partial recovery at the 24 h time point), while gastric leptin levels remain unchanged after 8 h fasting, indicative of their lower secretion when no food is ingested [44]. The gastric leptin levels are significantly decreased at the 24 h time point, probably reflecting the previous significant drop in their mRNA levels. Thus, both changes in adipose derived and gastric leptin, either by circulation or by vagal influence [8, 9], could be related to the changes observed in hypothalamic neuropeptide expression. Moreover, the results suggest that the regulation of leptin mRNA levels is a process that happens relatively soon in response to fasting. Also, it has to be pointed out that, differently to other studied parameters, the recovery in leptin mRNA and circulating leptin levels with refeeding is relatively slow and 3 h refeeding after 8 h fasting are, in general, not enough to reach the control levels, except for gastric mRNA levels and iWAT mRNA levels and with a tendency to recover for rWAT mRNA. Notably, the lack of recovery with refeeding in gastric leptin levels (considering the rise in the mRNA levels) would reflect its secretion in response to food intake, according to its role as a satiety signal [8, 11, 44]. Curiously, gastric leptin levels were positively correlated with circulating insulin levels ($r=0.363$, $p<0.05$) and this may suggest the possibility that gastric leptin levels may depend, at least in part, on circulating insulin levels; along these lines, not only has adipose leptin expression been clearly shown to be induced by insulin [45-47], but it also happens for gastric leptin as shown in human gastric cultured cells [48].

Although it is well established that the expression of NPY/AgRP and of POMC/CART in the hypothalamus are increased and decreased respectively in situations of negative energy balance [1, 3, 4, 7, 13], we show here that short periods of fasting cause only the induction of the mRNAs of the orexigenic (NPY/AgRP) neuropeptides, without differences in the anorexigenic (POMC/CART) ones. Thus, it is suggested that the NPY/AgRP neurons may be highly sensitive to the hormonal and metabolite changes associated to food deprivation.

Fig. 4. Simple correlation analysis of hypothalamic AgRP (agouti related peptide) mRNA levels with circulating leptin (A), insulin (B) and glucose (C). mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of animals under *ad libitum* feeding conditions. The Pearson's correlation index is given together with the threshold of significance. Data comes from the analysis of $n = 26$ -28 animals.

The elevation in NPY mRNA levels observed here is transient (only significant at the 4 h fasting time-point), but can be important, if translated to increased protein levels and secretion, in initiating/promoting the pro-orexigenic signaling by regulating “second order” neurons. Later increases of NPY expression are also expected, as has been reported before with longer periods of fasting [49], and the transient elevation in NPY expression observed here, at relatively short times of fasting, can be related to a necessary, but buffered, orexigenic response to food deprivation that would only be more powered if fasting persisted for a longer time. On the other hand, the early and sustained elevation of AgRP mRNA highlights the importance of AgRP in food intake regulation. It has been previously shown that while the contribution of the melanocortin receptor agonist α -MSH (whose precursor POMC mRNA levels do not change here) is important, it is the antagonist AgRP whose mRNA shows a greater degree of regulation by extremes of negative or positive energy balance and by genetic leptin deficiency [6, 13], which is in accordance with our results. Moreover, NPY has been described to have a significantly shorter duration of action within the arcuate to second order neurons projection than AgRP; thus, although the signaling properties of NPY and AgRP may be viewed as redundant, the difference in duration of action indicates potential differences in effect [6].

Different signals regulated during fasting may be responsible for the changes in NPY/AgRP expression observed here and, in fact, the neurons producing them can act not only as responders to leptin levels (as is well established), but also as sites where more than one type of energy homeostasis related signals could be integrated [6], such as insulin and glucose levels. The NPY and AgRP mRNA induction agrees with the reported effects of leptin, insulin and glucose on their expression: the neurons that produce them express leptin and insulin receptors and a drop in the levels of these hormones activates the NPY/AgRP neurons [3, 9, 13, 19, 21, 36]; also, the decrease in blood glucose associated to food deprivation is clearly linked to the increased expression of NPY/AgRP [36]. Different signaling pathways may

be modulated in the hypothalamic neurons by changes in circulating leptin, insulin and glucose, and all these signals can converge and regulate the expression of the neuropeptides by the JAK-STAT [9, 3], PI3K [20, 21], AMPK (AMP activated protein kinase) and malonyl-CoA related signaling pathways, and by altering firing patterns of the neurons [3].

Thus, changes in these signals, by integrating all these pathways, are candidates to explain the changes observed here for AgRP expression, although they may have different relative importance in the short term periods of fasting and two considerations must be made. First, a correlation analysis carried out (see figure 4) points out the significant negative correlation of AgRP mRNA levels in the hypothalamus with the circulating levels of both leptin (4A) and insulin (4B), and also with glucose levels, although less significantly (4C); this is in accordance with the demonstrated effects of these signals on AgRP neurons [3, 9, 13, 19, 21, 36]. Second, 3 hours of refeeding

after 8 hours of fasting is not able to provoke a decrease in the elevated AgRP mRNA levels and the only circulating signal of the ones analyzed that does not rise with refeeding up to control levels is leptin. Therefore, it can be suggested that, although insulin signaling seems to be of importance in the fasting condition for the hypothalamus, leptin signaling may be of crucial importance in short term fasting and refeeding in regulating the expression of orexigenic neuropeptides. On the other hand, in our analysis, NPY mRNA levels are not correlated with any of these parameters, but this is logical since they suffered a transitory elevation and all the time points have been considered in the correlation analysis. Besides, we must consider that we analyzed the neuropeptide mRNA expression in the whole hypothalamus and, while the expression of AgRP is confined to a small population of cells in the arcuate, NPY is also expressed in other hypothalamic neuron populations [4]; thus, this is another fact that can be related with the lack of correlation between NPY expression with both circulating leptin and insulin.

Further studying the leptin and insulin signaling pathways, it is reported here that the mRNA of the long form of the leptin receptor (OB-Rb mRNA) is significantly elevated at the time points of 8 and 24 hours of fasting, while SOCS3 and InsR mRNAs do not change their levels. Although leptin signaling is decreased with fasting due to the drop in leptin levels, curiously the leptin receptor responds by increasing its expression; we hypothesize that this up-regulation may help buffer the orexigenic

stimulus by increasing the hypothalamic sensitivity to leptin and also allow the neurons to be prepared for a more sensitive response to a rise in leptin levels when fasting finishes. Also, these results point out the importance of the regulation of leptin signaling in the hypothalamus with fasting.

In summary, the results reported here suggest that NPY/AgRP producing neurons would be early responders to fasting, with a transient induction in NPY mRNA expression and a sustained induction of AgRP expression, highlighting the importance of the orexigenic stimulus at the beginning of food deprivation. Moreover, changes in different signals such as leptin, insulin and glucose levels may be of importance in regulating NPY and AgRP expression during fasting, with an outstanding role for leptin, in accordance with one of its main physiological roles, which is to combat the threat of starvation and the depletion of energy stores in mammals.

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