Pro-TRH and pro-CRF expression in paraventricular nucleus of small litter-reared fasted adult rats

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Abstract

Neuroendocrine axes adapt to nutrient availability. During fasting, the function of the hypothalamus–pituitary–thyroid axis (HPT) is reduced, whereas that of the hypothalamus–pituitary–adrenal axis (HPA) is increased. Overfeeding-induced hyperleptinemia during lactation may alter the regulatory set point of neuroendocrine axes and their adaptability to fasting in adulthood. Hyperleptinemia is developed in rodents by litter size reduction during lactation; adult rats from small litters become overweight, but their paraventricular nucleus (PVN) TRH synthesis is unchanged. It is unclear whether peptide expression still responds to nutrient availability. PVN corticotropin-releasing factor (CRF) expression has not been evaluated in this model. We analyzed adaptability of HPT and HPA axes to fasting-induced low leptin levels of reduced-litter adult rats. Offspring litters were reduced to 2–3/dam (early-overfed) or maintained at 8/dam (controls, C). At 10 weeks old, a subset of animals from each group was fasted for 48 h and leptin, corticosterone, and thyroid hormones serum levels were analyzed. In brain, expressions of leptin receptor, NPY and SOCS3, were evaluated in arcuate nucleus, and those of proTRH and proCRF in PVN by real-time PCR. ProTRH expression in anterior and medial PVN subcompartments was assayed by *in situ* hybridization. Early-overfed adults developed hyperphagia and excessive weight, together with decreased proTRH expression in anterior PVN, supporting the anorexigenic effects of TRH. Early-overfed rats presented low PVN proTRH synthesis, whereas fasting did not induce a further reduction. Fasting-induced stress was unable to increase corticosterone levels, contributing to reduced body weight loss in early-overfed rats. We concluded that early overfeeding impaired the adaptability of HPT and HPA axes to excess weight and fasting in adults.

Key Words

- ▶ PVN
- ▶ TRH
- ▶ CRF
- ▶ fasting
- ▶ hyperleptinemia
- ▶ early overnutrition

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Introduction

Adaptation of the functioning of neuroendocrine axes allows humans and animals to face environmental challenges such as food availability or stressful conditions by saving energy for the display of defensive actions or by

reducing their metabolic rate during food deficit. This adaptability may be compromised in adults subjected to stress, malnutrition (Ravelli *et al.* 1976, Parsons *et al.* 2001) or overfeeding during prenatal and early post-natal

periods (Rodrigues *et al.* 2009), thus enhancing the risk for the development of different metabolic disturbances.

Our aim was to evaluate the responsiveness of neurons in the paraventricular nucleus (PVN) that express the neuropeptides, pro-thyrotropin-releasing hormone (proTRH) and pro-corticotropin-releasing factor (proCRF), which regulate the hypothalamus–pituitary–thyroid (HPT) and hypothalamus–pituitary–adrenal (HPA) axes. This responsiveness was assessed after the known 48 h fasting-induced shift in leptin and corticosterone-circulating levels in early-overfed animals. In particular, we propose the hypothesis that the function of HPT and HPA axes would be compromised in adults by litter size reduction during lactation, when they are subject to 48-h period of fasting.

Nutrient availability is capable of modifying the functioning of HPT and HPA axes. HPT maintains energy homeostasis and regulates thermogenesis and basal metabolic rate by controlling the serum concentration of thyroid hormones (TH) – triiodothyronine (T₃) and thyroxine (T₄) – as well as their degrading effects on fuel reservoirs. TRH is the hypophysiotropic factor that controls HPT axis function. This peptide is synthesized in the medial PVN (mPVN) of the hypothalamus and released into the portal blood to stimulate thyrotropin (TSH) synthesis in the adenohypophysis and regulate its blood content. TSH in turn induces TH production in the thyroid gland (Nillni 2010). The HPA is driven directly by PVN CRF through the stimulation of corticotropin (ACTH) release from the adenohypophysis, which in turn induces corticosterone release from the adrenal cortex.

Fasting and prolonged food restriction decrease T₃ serum levels and mPVN proTRH mRNA content despite the negative feedback that TH generally exerts on TRH synthesis (Blake *et al.* 1991). TRH downregulation is, at least in part, due to low circulating leptin levels induced by negative energy balance, along with high corticosterone serum content. This fasting-induced shift in circulating hormone concentration activates type 2 deiodinase (D2) enzymatic activity in the median eminence (Coppola *et al.* 2005a), which in turn increases local T₃ content in the hypothalamus, inhibiting proTRH expression. In contrast, hyperphagia and being overweight lead to high leptin and T₃ serum levels in humans (Reinehr 2011) and rodents (Perello *et al.* 2010), as well as increased PVN proTRH expression in adult offspring of dams fed a high-fat diet (Franco *et al.* 2012). These manifestations act as counter-regulatory mechanisms in response to high body adiposity. ProCRF expression and the HPA axis are activated by fasting-induced stress. As a consequence circulating levels of ACTH and corticosterone are increased.

If the litter size is reduced to 3 pups/dam, rodents become overfed and develop hyperleptinemia and hyperthyroidism starting at weaning (Rodrigues *et al.* 2009). In adulthood, they are hyperphagic and overweight in comparison to offspring from litters of 8 to 10 pups/dam (Plagemann *et al.* 1992, Lopez *et al.* 2005, Rodrigues *et al.* 2009). Leptin serum levels are increased or normal in early-overfed adult rats (Lopez *et al.* 2005, Rodrigues *et al.* 2009), proTRH mRNA content in the PVN is unchanged (Lopez *et al.* 2007). Thus, it is difficult to determine whether the HPT axis of these animals is still responding to changes in serum leptin and corticosterone levels and to nutrient availability. ProCRF expression in the PVN of early-overfed adult rats has not been described, whereas corticosterone serum levels are normal (Boullu-Ciocca *et al.* 2005). Normal values of PVN proTRH expression and corticosterone and leptin serum levels of early-overfed adults do not ensure that neuroendocrine axes can respond to a new metabolic challenge as those of control animals do.

In order to induce a metabolic challenge, we subjected early post-natal overfed adult rats to a 48-h period of fasting and evaluated both expression changes of proTRH mRNA content in the hypophysiotropic neurons of the PVN and HPT adaptation to low circulating levels of leptin and high circulating levels of corticosterone. In addition, we evaluated whether proTRH mRNA levels in the anterior PVN (aPVN) changed in a manner correlated with the hyperphagia of animals, since the peptide from this subcompartment has been implicated in feeding regulation (Wittmann *et al.* 2009a, Alvarez-Salas *et al.* 2012). To identify the functioning of the transduction pathway of leptin in adults, we also evaluated expression patterns of target genes of leptin signaling in the arcuate nucleus (ARC) and PVN, such as *Lepr* and *Npy*, which directly or indirectly affect PVN proTRH mRNA levels during fasting.

We also analyzed changes in PVN proCRF mRNA levels of both animals allowed to feed *ad libitum* and fasted animals from normal or small litters, since an adequate degradation of fuel deposits by corticosterone ensures enough energy availability for brain and basal functioning of the organism, and we wanted to elucidate whether this process was adequately achieved by overfed animals.

Materials and methods

Animals

All experiments were approved by the local committee of ethics on animal experimentation and complied with the

Mexican official norm NOM-062-ZOO-1999 relating to experiments with laboratory animals.

Pregnant female Wistar rats ($n=10$; 300–350 g) (INPRFM animal house) were individually housed at 25 °C and maintained on a 12 h light:12 h darkness cycle, with free access to standard laboratory rat chow (Lab rodent diet no. 5001; PMI Feeds, Brentwood, MO, USA) and tap water. To induce early post-natal over-feeding, on lactation day 3, litter size for six dams was reduced to two to three male pups, while normal litter groups were formed by four dams with eight pups (males and females) per litter.

A set of males from each size of litter (8/litter size group) was killed at weaning. The remaining males were assigned to four groups: i) control (C, $n=7$) whose litter was normal sized; ii) early overfed from reduced litters ($n=5$). Both groups were allowed to feed *ad libitum*; iii) fasted controls (FC, $n=8$); and iv) fasted overfed (F-overfed, $n=5$); rats were fasted 48 h before they were killed. Body weight and food intake were measured weekly, starting at weaning and continuing until 10 weeks of age, when they were killed by decapitation. The experiment was repeated twice, thus the final number of animals per group was 18 in average. Trunk blood was collected to measure T_3 , T_4 , and corticosterone in adults, and leptin in weaned and adult animals. Abdominal (mesenteric and retroperitoneal) white adipose tissue in adults was excised and weighed. Brains of adults were extracted and stored at –70 °C until processed. Changes in expression of different genes were determined by quantitative real-time PCR on ARC and PVN on one half of the animals; proTRH mRNA levels in PVN ($n=8$) were analyzed by *in situ* hybridization (ISH) on the other half.

Quantitative real-time PCR analysis

Frozen PVN and ARC were punch-dissected using a 1 µm diameter sample corer and homogenized in 4 M guanidine thiocyanate (ICN, Aurora, OH, USA). Total RNA was extracted as described elsewhere (Chomczynski & Sacchi 1987). RNA quality of samples was verified by the O.D. reading of the 28s:18s ratio from an agarose gel electrophoresis; samples were discarded when the ratio was not higher than 1.5. Briefly, 1.5 µg of RNA and oligo-dT (100 pmol/µl) (Biotechnology Institute facilities, UNAM, Mexico) were used to obtain cDNA (M-MLV reverse transcriptase (Invitrogen)). PVN proTRH, proCRF, ObRb, and SOCS3, as well as ARC NPY, SOCS3, and ObRb receptor mRNA levels were quantified by real-time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems)

on a 7500 real-time PCR system. Oligonucleotide primers used were from Applied Biosystems, viz.: *Trh*, Rn00564880_m1; *Crf*, Rn01462137_m1; *Npy*, Rn01410145_m1; *Lepr*, Rn01433205_m1; *Socs3*, Rn00585674_s1; *Actb*, Rn00667869_m1, and *Gapdh*, Rn99999916_s1. Each PCR was performed in duplicate. mRNA expression levels for each gene examined were normalized using the geometric mean of the expression of β -actin (*Actb*) and GAPDH (*Gapdh*) taken as controls, as described (Vandesompele *et al.* 2002). Changes in mRNA content were evaluated from the threshold cycle. The ΔC_t value was obtained from the difference between the number of cycles for the target genes and that for the endogenous genes to reach the threshold ($\Delta C_t = C_t \text{ unknown} - C_t \text{ geometric mean of } Actb \text{ and } Gapdh$). The percentage of change for each gene examined was obtained by the equation: $\Delta\Delta C_t = 2^{-(\Delta C_t = C_t \text{ Control} - C_t \text{ Overfed group})} \times 100$.

Tissue treatment for ISH histochemistry

Brains stored at –20 °C were sectioned using a cryostat (Microm HM 525; Carl Zeiss IMT Corp., Maple Grove, MN, USA). The resulting 14-µm coronal slices were adhered to Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Sections through the whole extension of the PVN were collected (aPVN comprised from –1.1 to –1.5 mm from bregma, and mPVN from –1.5 to –2.3 mm from bregma) (Paxinos & Watson 2005). ProTRH expression was analyzed in two different PVN subcompartments because that of the aPVN is implicated in feeding regulation, and that of the mPVN in the regulation of HPT axis function. As there is no available evidence showing that proCRF is differentially expressed in aPVN or mPVN in feeding and HPA axis regulation, we did not perform ISH analysis of proCRF expression. The sections were fixed with 4% paraformaldehyde at 4 °C followed by digestion with pronase (24 U/ml) at 20 °C. The slides were air-dried before hybridization.

ISH histochemistry

Oligonucleotides were used as ISH probes. The following sequences directed against proTRH mRNA (GenBank accession no. NM_013046.2) were labeled by 3'-tailing with [35 S]dATP (1250 Ci/mmol; PerkinElmer, Waltham, MA, USA) or with Dig-11-dUTP using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH): i) 5'-TGC CCA TGA ATA CTT GTC CTG GTT GGC ACG TCG GCC GGG GTG CTG-3'; ii) 5'-ATC AGA CTC CAT CCA GGG GAA GGA TCG CCT GCC AGG GTG CTG CCG-3'; iii) 5'-AGG

GTG AAG ATC AAA GCC AGA GCC AGC AGC AAC CAA GGT CCC GGC-3'. The ISH protocol has been described elsewhere (de Gortari & Mengod 2010). Specificity of the oligonucleotides was verified by the unique distribution pattern of the proTRH signal similar to that previously described (Segerson *et al.* 1987). Also, we observed no signal in the PVN when slices were hybridized with sense sequences of the same oligonucleotides and labeled with digoxigenin (Fig. 1). Briefly, hybridizations were performed by incubating slides overnight at 42 °C with buffer containing 50% formamide/4× SSC, 10% dextran sulfate, 1× Denhardt's solution (0.02% BSA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone), 1% sarkosyl, 20 mM phosphate buffer (pH 7.0), 250 µg/ml yeast tRNA, 500 µg/ml denatured salmon sperm DNA, 5 M dithiothreitol, and 10–20×10⁶ c.p.m. radiolabeled oligonucleotide probe per milliliter of hybridization buffer or 4 pmol of dig-labeled sense or antisense probes per milliliter of hybridization buffer. Slides were washed four times at 60 °C with a buffer containing 0.6 M NaCl and 10 mM Tris-HCl (pH 7.5), and those hybridized with radiolabeled probes were subsequently dipped into Kodak NTB autoradiography emulsion (Eastman Kodak). Autoradiograms were developed with Kodak-D19 after a 3-week exposure at 4 °C. Following the washes (0.6 M NaCl, 10 mM Tris-HCl pH 7.5), brain sections hybridized with sense and antisense dig-labeled probes were immersed in a buffer containing 0.1 M Tris-HCl pH 7.5, 1 M NaCl, 2 mM MgCl₂, and 0.5% BSA

(Sigma–Aldrich) and then incubated overnight at 4 °C in the same buffer containing alkaline-phosphatase-conjugated anti-digoxigenin-F(ab) fragments (1:5000) (Roche Diagnostics GmbH). The same solution but without the antibody was used to wash the slides three times for 10 min/each, followed by two washes in an alkaline buffer that contained 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 5 mM MgCl₂. Development of alkaline phosphatase activity was achieved by incubating sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Diagnostics GmbH) diluted in 10 ml of alkaline buffer. Thorough rinses in the alkaline buffer containing 1 mM EDTA were used to stop the enzymatic reaction. Finally, the slides were dehydrated rapidly in 70 and 100% ethanol, air-dried, and mounted in 50% of glycerol in PBS solution.

Image analysis

Signal from the autoradiograms was visualized using an Olympus BH-2 imaging microscope (New York Microscope Co., Inc., Hicksville, NY, USA) under dark-field illumination. Images were captured with a 4× objective using an Evolution MP Media Cybernetics video camera (Media Cybernetics, Inc., Bethesda, MD, USA) and analyzed using Image-Pro Plus 7.0 computer software (Media Cybernetics, Inc.). For each animal, integrated O.D. values (with background correction) were measured in six rostrocaudal slices which spanned both PVN subdivisions of interest.

Figure 1

Bright-field illumination photomicrographs of sections from the medial subdivision of hypothalamic PVN after *in situ* hybridization with digoxigenin-labeled antisense (A) and sense (B) probes directed against

proTRH mRNA. Note the lack of specific signal in the PVN with the sense probes. III, third ventricle. Scale bar: 100 µm.

Analysis of hormone serum levels

Serum total T_3 concentration was determined by ELISA (Alpco Diagnostics Kit, Salem, NH, USA); sensitivity: 20 ng/dl, inter- and intra-assay variability: 4.9 and 5.6% respectively. Serum corticosterone concentration was assessed by RIA (Coat-A-Count Rat Corticosterone I^{125} TKRC1, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA); sensitivity 5.7 ng/ml, inter- and intra-assay variation: 8.5 and 6.8% respectively. Circulating leptin concentration was determined colorimetrically with an ELISA kit for rat (Assay Designs, Ann Arbor, MI, USA); sensitivity: 0.672 ng/ml, inter- and intra-assay variability: 7.2 and 5.9% respectively. Total T_4 was also determined by ELISA (Alpco Diagnostics Kit); sensitivity: 0.5 μ g/dl, inter and intra-assay variability: 3.6 and 3.8% respectively.

Statistical analysis

A Student's *t*-test was performed to compare body weight and food intake between control and early-overfed adult groups on each week of the experiment. A two-way ANOVA was performed to analyze differences between animals allowed to feed *ad libitum* and fasted adult animals. A one-way ANOVA was used for all parameters in weaned pups. Before the performance of variance analyses, we confirmed the Gaussian distribution of the data. A Pearson correlation was performed between PVN TRH mRNA levels and corticosterone and leptin serum concentrations. When the difference was significant ($P < 0.05$), the analysis was followed by Fisher's *post-hoc* test.

Results

Body and white adipose tissue weights

Early-overfed weaned animals had a body weight 20% higher than that of control offspring, and with the exception of week 7, it remained increased up to the end of the experiment. Differences between groups were analyzed by Student's *t*-test each week (Fig. 2A). Abdominal fat weight was higher in the early-overfed group by 21% (3.8 ± 0.2 g) vs controls (3.16 ± 0.2 g, 100%). After fasting, FC groups showed a decrease by 33% (2.4 ± 0.16 g) vs controls whereas in the F-overfed group abdominal fat was only reduced by 18% (3.1 ± 0.1 g), but still was different from that of the early-overfed group. A two-way ANOVA showed a significant effect of group: $F_{(1,10)} = 23.23$, $P < 0.001$ and diet: $F_{(1,10)} = 46.216$, $P < 0.0001$ but no significant interaction between variables $F_{(1,10)} = 0.091$, $P > 0.05$.

Figure 2

Cumulative body weight and food intake. (A) Body weight values are the weekly body weight registers of control and early-overfed groups starting at weaning and up to week 10. Data are expressed as the mean $\pm$ S.E.M. in grams ($n = 16$ /group). (B) Food intake values are the weekly average of the daily food intake of controls and early post-natal overfed groups starting at weaning and are expressed as the mean $\pm$ S.E.M. ($n = 16$ /group). * $P < 0.05$

Body weight loss after fasting

F-overfed adult animals lost 11% of their body weight, whereas the FC group lost 14.3% of the body weight compared with their respective controls. The difference was significant when a Student's *t*-test was performed: $t = 2.189$, $P < 0.05$.

Food intake

After weaning, early-overfed animals ate more food than controls starting on week 5 (4%) and up to the end of the experiment (11% on average); differences between groups were evaluated by Student's *t*-test every week (Fig. 2B).

Serum hormones

Leptin Weaned early-overfed animals exhibited a 116% increase in serum leptin levels vs those of the control group ($C = 1.4 \pm 0.2$; early overfed = 3.1 ± 0.3 ng/ml, $F_{(1,18)} = 20.922$, $P < 0.001$), whereas in adults, there was no difference in leptin concentration between

experimental and control groups. Fasting reduced leptin levels to undetectable values both in FC and F-overfed animals (Table 1). A two-way ANOVA showed a significant effect of diet: $F_{(1,18)}=18.54$, $P<0.001$ and no significant effect of group: $F_{(1,18)}=0.486$, $P>0.05$ or interaction between variables: $F_{(1,18)}=0.093$, $P>0.05$.

Corticosterone Control and early-overfed adults did not differ in serum corticosterone content; FC showed an increase of 92% and F-overfed a decrease to 37% vs their respective controls allowed to feed *ad libitum*. A two-way ANOVA showed a significant effect of groups: $F_{(1,32)}=5.740$, $P<0.05$ and interaction between variables: $F_{(1,32)}=5.791$, $P<0.05$ (Table 1).

Triiodothyronine Controls and early-overfed adults presented similar T_3 serum content; fasting reduced levels of this hormone by 25% in FC and by 17% in F-overfed vs their respective controls that had been allowed to feed *ad libitum*. A two-way ANOVA showed a significant effect of diet: $F_{(1,70)}=12.581$, $P<0.001$, no significant effect of group: $F_{(1,70)}=0.043$, $P>0.05$, or interaction between variables: $F_{(1,70)}=1.955$, $P>0.05$ (Table 1).

Thyroxine Early-overfed adults showed a slight reduction of 16% in T_4 circulating levels vs controls. After fasting, FC and F-overfed groups exhibited 32 and 21% lower T_4 than their respective groups allowed to feed *ad libitum*. There was a significant effect of group: $F_{(1,16)}=8.6$, $P<0.01$, diet: $F_{(1,16)}=69.63$, $P<0.0001$, and interaction between variables: $F_{(1,16)}=4.888$, $P<0.05$ (Table 1).

Table 1 Serum hormone concentrations. Serum hormone content was evaluated in weaned adult animals allowed to feed *ad libitum* or fasted. The number of animals for T_3 determination was 18/group; for T_4 , $n=5$ /group, and for corticosterone and leptin, $n=10$ /group. Values are the mean $\pm$ S.E.M. and are expressed in ng/ml for leptin and corticosterone; ng/dl for T_3 ; μ g/dl for T_4 . Differences were found by two-way ANOVA between data of groups and diets followed by Fisher's *post-hoc* test, when differences were significant

	C	Overfed	FC	F-overfed
T_3 (ng/dl)	100 $\pm$ 4	98 $\pm$ 4	75 $\pm$ 4.8* [†]	83 $\pm$ 5.7*
T_4 (μ g/dl)	14 $\pm$ 0.3	11.9 $\pm$ 0.4*	9.6 $\pm$ 0.35*	9.3 $\pm$ 0.5 [†]
Leptin (ng/ml)	2 $\pm$ 0.3	1.67 $\pm$ 0.2	ND	ND
Corticosterone (ng/ml)	99 $\pm$ 20	102 $\pm$ 35	192 $\pm$ 45* [†]	37 $\pm$ 5* [†]

C, control; FC, fasted-control; F-overfed, fasted-overfed; ND, not-detectable values. * $P<0.05$ compared to C; [†] $P<0.05$ vs early-overfed.

Changes in hypothalamic mRNA peptide expression

ARC ObRb expression was higher in ARC for all experimental groups – early-overfed, FC, and F-overfed – compared with controls but no difference was found between experimental groups. ANOVA showed a significant effect of diet: $F_{(1,10)}=15.09$, $P<0.01$ and interaction between variables: $F_{(1,10)}=20.017$, $P<0.01$ (Fig. 3). SOCS3 mRNA content increased in FC and F-overfed vs controls. The highest expression was found in the F-overfed group which was significantly different from the other three groups. ANOVA showed a significant effect of group: $F_{(1,8)}=10.67$, $P<0.05$, diet: $F_{(1,8)}=25.9$, $P<0.001$, and interaction between variables: $F_{(1,8)}=5.71$, $P<0.05$ (Fig. 3). NPY mRNA content increased only in the FC group vs controls. A two-way ANOVA yielded a significant effect of group: $F_{(1,8)}=12.014$, $P<0.01$, diet: $F_{(1,8)}=11.815$, $P<0.01$ and interaction between variables: $F_{(1,8)}=9.584$, $P<0.05$ (Fig. 3).

Paraventricular nucleus ObRb and SOCS3 expression did not change in any of the groups. ProTRH mRNA levels decreased in all experimental groups vs controls; reduction of proTRH expression in the early overfed group was greater than the one induced by fasting in the FC group. A two-way ANOVA showed a significant effect of group: $F_{(1,11)}=37.11$, $P<0.0001$, diet: $F_{(1,11)}=8.45$, $P<0.05$ and interaction between variables: $F_{(1,11)}=20.454$, $P<0.001$ (Fig. 3). Correlations between TRH mRNA levels and those of corticosterone and leptin concentrations in C rats resulted significantly negative ($r=-0.917$, $P<0.05$) and positive ($r=0.88$, $P<0.05$), respectively, but no correlation was found in early-overfed animals (cort: $r=-0.68$, $P>0.05$; leptin: $r=-0.49$, $P>0.26$). ProCRF expression was also lower in the three experimental groups when compared with controls; ANOVA showed a significant effect of group: $F_{(1,10)}=21.012$, $P<0.01$; diet: $F_{(1,10)}=5.313$, $P<0.05$, and interaction between variables: $F_{(1,10)}=5.754$, $P<0.05$ (Fig. 3).

ProTRH expression in PVN subcompartments by ISH

The early-overfed group exhibited a reduction to 35% in proTRH expression in the aPVN, whereas in FC it decreased to 42% when compared with controls (100%). The F-overfed group did not show any difference vs C. A two-way ANOVA showed a significant interaction between diet and group: ($F_{(1,21)}=9.7$, $P<0.01$) (Figs 3 and 4).

In the mPVN, early-overfed animals showed a decrease of 47.8% in proTRH mRNA levels vs controls. Fasting also induced a decrease to $\sim 47\%$ in both FC and F-overfed groups vs controls allowed to feed *ad libitum* (100%).

Figure 3

Peptide mRNA content in arcuate and paraventricular nuclei of adult animals, analyzed by quantitative real-time PCR. Values are mRNA expression of each examined amplicon (for ARC: ObRb, SOCS3, NPY; for PVN: ObRb, SOCS3, proTRH and proCRF) after being normalized against a factor consisting in the geometric mean of the expression of two reference

genes: *Actb* and *Gapdh*, and expressed as the mean $\pm$ S.E.M. of the % of controls (100%) ($n=5$ /group). A two-way ANOVA was performed to find differences between groups and diets of adult animals: * $P<0.05$ vs C (controls); # $P<0.05$ vs FC (fasted-controls); & $P<0.05$ vs F-overfed (fasted-overfed).

A two-way ANOVA showed a significant effect of group: ($F_{(1,26)}=7.324$, $P<0.05$), diet: ($F_{(1,26)}=7.2$, $P<0.05$), and interaction between variables: ($F_{(1,26)}=7.237$, $P<0.05$) (Figs 4 and 5).

Discussion

During critical stages of development, alterations in serum hormone concentrations involved in energy balance regulation, as well as high glycemia due to overfeeding or to a diabetic dam, impair the ability of neuroendocrine axes to adapt to metabolic or stressful challenges in adult humans and animals. These early disturbances may put the survival of an organism at risk or increase its susceptibility to developing pathologies such as the

metabolic syndrome later in life. The aim of this study was to evaluate the adaptability of HPT and HPA axes to a 48-h period of fasting in adult animals that presented hyperleptinemia and overweight during lactation due to litter size reduction.

Early-overfed adult rats allowed to feed *ad libitum* exhibited similar changes to those previously observed by others regarding body weight, leptin levels, and mRNA expression of ObRb and NPY in the arcuate nucleus (Lopez *et al.* 2005, 2007, Rodrigues *et al.* 2011). In addition, the expected hyperleptinemia and overweight were present in weaned early-overfed animals.

Body weight and food intake in the early-overfed group were higher than those of controls starting at weaning and up to the tenth week of age, as described

Figure 4

ProTRH mRNA expression in PVN subcompartments determined by ISH. Changes in proTRH mRNA levels in aPVN and mPVN of control (C), early-overfed, control animals fasted for 48 h (FC) and animals from small litters fasted for 48 h (F-overfed) animals quantified by ISH

histochemistry. Rats were killed at 10 weeks of age. Values are expressed as the mean $\pm$ S.E.M. of the percentage for controls (100%) of integrated O.D. * $P<0.05$ vs C; & $P<0.05$ vs F-overfed.

Figure 5

Dark-field illumination photomicrographs of representative samples of proTRH mRNA in PVN. Images are low-power magnifications of anterior (1) and medial (2) subdivisions of the PVN of control group (A),

early-overfed group (B), control animals fasted for 48 h (FC) group (C) and animals from small litters fasted for 48 h (F-overfed) group (D). Scale bar, 200 μ m.

previously (Davidowa *et al.* 2003, Boullu-Ciocca *et al.* 2005). Increased body weight and white adipose tissue mass of early-overfed adults did not correlate with their circulating leptin levels, which remained similar to those of controls. A plausible explanation for this are the basal corticosterone levels found in early-overfed rats, which were similar to control values. It is commonly observed that circulating glucocorticoid concentrations are higher in overweight humans and animals; glucocorticoids are well-known secretagogues of leptin (Hardie *et al.* 1996, Russell *et al.* 1998, Williams *et al.* 2000). As we discuss below, we found a deregulated adrenal axis in early-overfed adult rats that could contribute to the unchanged circulating leptin levels in this group. Rodrigues *et al.* (2011) have also reported leptin levels similar to controls in early-overfed groups using the same model.

We found enhanced ObRb and SOCS3 expression in the ARC of early-overfed animals, supporting the resistance of this nucleus to the effects of leptin, as has been suggested by other authors (Rodrigues *et al.* 2011). SOCS3 is a negative regulator of the leptin signaling cascade; hence its higher expression may impair the positive and negative effects of leptin on the synthesis of anorexigenic and orexigenic peptides respectively. However, NPY expression was not different in early-overfed compared with control adults, as has been described previously (Lopez *et al.* 2007), which could argue against leptin resistance in the early-overfed animals allowed to feed *ad libitum*. As this group showed serum leptin levels in the

normal range, we needed to challenge the ARC response to fasting-induced hypoleptinemia to confirm a blocked leptin signaling. We also observed basal values of glucocorticoids in the early-overfed group; these compounds are known to stimulate NPY synthesis in the ARC (White *et al.* 1990, Sato *et al.* 2005, Goto *et al.* 2006), thus by increasing serum glucocorticoid levels after fasting we could possibly identify the response of NPY-expressing neurons to another peripheral signal of energy status.

TRHergic neurons implicated in hypophysiotropic functions are only those of the medial parvocellular part of the PVN (Wittmann *et al.* 2009b). As a consequence, we evaluated proTRH expression in this subcompartment to assay neurons driving the function of the HPT axis. T₄ serum content is slightly decreased in the early-overfed group according to Plagemann *et al.* (1999) and we found that proTRH expression in the mPVN subcompartment was also lower than that in controls. Since early-overfed adult rats were overweight, the HPT axis must be activated, as is observed in obese humans and in some obesity models in rodents (Reinehr & Andler 2002, Perello *et al.* 2010). However, the early-overfed group exhibited an altered flexibility of the HPT axis to increased adiposity, which might induce, in the long term, deceleration of the metabolic rate, and favor the development of obesity and its complications. These results indicate that central hypothyroidism may be the cause of the slightly low TH serum content found in overweight early-overfed adults.

PVN TRHergic cells are innervated by ascending catecholaminergic neurons from the brainstem (Cunningham & Sawchenko 1988, Füzesi *et al.* 2009). Noradrenaline (NA) is an upregulator of hypophysiotropic proTRH expression (Uribe *et al.* 1993, Perello *et al.* 2007). Importantly, brain NA concentrations in early-overfed animals are low (Seidler *et al.* 1990), and the binding of adrenergic receptors in PVN is reduced in diet-induced obese rats (Levin 1996). This is also indicated by the reduced catecholamine release in caffeine-stimulated adrenal cells of early-overfed adult rats produced by litter reduction, as well as by low expression of the β 2-adrenergic receptor evaluated in liver homogenates, which may lead to decreased peripheral sensitivity to catecholamines and lipolysis rate (Conceicao *et al.* 2013). Thus, NA is a candidate inhibitor of TRH synthesis in the mPVN, as well as that of CRF as described below.

TRH-expressing neurons of the aPVN do not project to the median eminence, but to other regions involved in feeding regulation, such as the dorsomedial and ARC hypothalamic nuclei (Wittmann *et al.* 2009a). In addition, proTRH expression in aPVN is increased in food-avoiding animals (Alvarez-Salas *et al.* 2012). We found that in this subcompartment proTRH mRNA levels were down-regulated in the early-overfed group, supporting the anorexigenic role of aPVN and, in part, the underlying hyperphagia of the early-overfed group. This is in agreement with early studies showing that PVN destruction leads to hyperphagia and overweight (Leibowitz *et al.* 1981, Fletcher *et al.* 1993). Leptin signaling in the PVN of the early-overfed group seemed functional considering the observed normal mRNA levels of ObRb and SOCS3; this is evident in the reduction of food intake of early-overfed adults receiving an i.c.v. injection of leptin (Lopez *et al.* 2007). Thus, we cannot discard the possibility that proTRH expression reduction in this group was more probably due to ARC feeding-regulatory neuropeptides, whose effects on PVN are altered by early post-natal hyperleptinemia (Davidowa *et al.* 2003). This hypothesis is likely given that aPVN and mPVN TRHergic neurons are innervated by axons containing α -MSH and AgRP receptors (Legradi & Lechan 1999, Fekete *et al.* 2000); but this issue needs to be elucidated.

PVN proCRF expression, which has anorexigenic effects (Arase *et al.* 1988), was decreased in the early-overfed group vs controls. As happened with proTRH from aPVN, this is also in accordance with the hyperphagic state of the early-overfed group. Early-overfed animals showed similar corticosterone serum levels to those of controls, as others have found (Boullu-Ciocca *et al.* 2005, Rodel *et al.* 2010, Bulfin *et al.* 2011). The decreased proCRF expression and

normal corticosterone serum levels of the early-overfed group are not congruent with the negative feedback regulation of the HPA axis exerted by glucocorticoid levels. Our results indicate (and were confirmed by the results for the fasted-overfed group) an altered regulation of the adrenal axis in early-overfed adults. This result supports the idea of an impaired afferent catecholaminergic pathway to the PVN in the overweight early-overfed group, because CRF is upregulated by NA (Cole & Sawchenko 2002). Interestingly, fetally fetal undernourished adult rats present high proCRF expression (Núñez *et al.* 2008), as well as enhanced brain noradrenergic synthesis and release (Soto--Moyano *et al.* 1998a,b).

Fasting

We evaluated the adaptability of the HPT axis to reduced leptin content induced by a 48-h period of fasting, which decreases body weight and represents a new metabolic challenge. The FC group displayed the expected changes induced by low nutrient availability: decreased leptin and increased serum corticosterone concentrations, whereas F-overfed animals showed reduced leptin levels but not the activation of the adrenal axis, confirming its deregulation in early post-natal overweight adult rats.

In ARC of the FC group, NPY, SOCS3, and ObRb synthesis were elevated, which is in agreement with the low leptin levels observed. These changes along with reduced proTRH expression in aPVN and mPVN were coincident with the expected stimulation of hypothalamic orexigenic and inhibition of anorexigenic peptides as a result of a negative energy balance. This reduced PVN proTRH expression could be due to the combined actions of high NPY release into this nucleus, as proposed by others (Legradi & Lechan 1999, Fekete *et al.* 2001), and supported here by high NPY synthesis in ARC. As mentioned earlier, it has also been proposed that T_3 local hypothalamic content increases by the action of D2 as a result of fasting-induced low circulating levels of leptin and high circulating levels of corticosterone (Coppola *et al.* 2005b). High hypothalamic concentrations inhibit TRH synthesis (Coppola *et al.* 2005a). This could not be the case however in F-overfed animals because they also presented peripheral hypothyroidism along with downregulation of proTRH in mPVN despite their low leptin and corticosterone serum levels, which may not be elevating local T_3 content (Coppola *et al.* 2005b). The absence of a correlation between serum corticosterone and leptin levels with PVN proTRH expression in the F-overfed group supports the hypothesis of an impaired response of TRHergic cells to

those hormones after fasting. These results are evidence of alternative factors capable of decreasing proTRH expression during fasting. We can discard the inhibitory effects of NPY on TRH synthesis, since its expression in ARC was normal in the overfed group. Furthermore, altered actions of NPY on PVN neurons have been described in early-overfed animals (Davidowa *et al.* 2003). Besides, the lack of an increase in ARC NPY expression in F-overfed rats revealed the impaired leptin signaling of this nucleus in early post-natal-overfed animals.

The low corticosterone content in the F-overfed group was surprising because during fasting its elevation facilitates degradation of lipid deposits and ensures glucose availability for brain and peripheral tissues. Thus, this was evidence of a disturbed HPA response to metabolic challenges in the F-overfed group that puts at risk the survival of animals facing a stressful event. Fasting activates NA afferent projections to PVN CRF-expressing neurons, stimulating CRF synthesis and release, which in turn enhances corticosterone serum levels. As part of the negative feedback, glucocorticoids downregulate PVN proCRF expression, which was evident here in the FC group. However, in F-overfed animals, decreased proCRF mRNA levels cannot be due to circulating levels of corticosterone since these were reduced in this group. The proposed impaired activation of NA connections to the PVN is supported by those changes in fasted early-overfed rats. Low corticosterone serum concentration may be avoiding degradation of energy stores by lowering body weight loss in the F-overfed group.

We showed here that early post-natal overfeeding and hyperleptinemia induced in animals by litter size reduction resulted in a long-lasting impairment in the adaptability of HPT and HPA axes to high adiposity and to a novel metabolic challenge such as fasting. Reduced expression of proTRH and proCRF in the PVN of early-overfed animals may contribute to their hyperphagia. These alterations diminished fuel deposit degradation and weight loss during low energy intake.

This animal model may be useful for understanding the refractoriness of adults with infant obesity or the offspring of diabetic mothers (Dussault *et al.* 1982, Walker & Courtin 1985, Pracyk *et al.* 1992, Cetin *et al.* 2000, Tapanainen *et al.* 2001, Wilcoxon & Redei 2004, Moura *et al.* 2008) to reduce weight in response to diet therapy and their propensity to develop obesity and its co-morbidities.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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