

Postnatal early overnutrition changes the leptin signalling pathway in the hypothalamic–pituitary–thyroid axis of young and adult rats

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Postnatal early overnutrition (EO) is a risk factor for obesity in adult life. Rats raised in a small litter can develop hyperinsulinaemia, hyperphagia, hyperleptinaemia and hypertension as adults. Since leptin regulates the hypothalamic–pituitary–thyroid axis and the metabolism of thyroid hormones, we studied the leptin signalling pathway in pituitary and thyroid glands of the postnatal EO model. To induce EO, at the third day of lactation the litter size was reduced to three pups per litter (SL group). In control litters (NL group), the litter size was adjusted to 10 pups per litter. Body weight and food intake were monitored. Rat offspring were killed at 21 (weaning) and 180 days old (adulthood). Plasma thyroid hormones, thyroid-stimulating hormone (TSH) and leptin were measured by radioimmunoassay. Proteins of the leptin signalling pathway were analysed by Western blotting. Body weight of offspring in the SL group was higher from the seventh day of lactation (+33%, $P < 0.05$) until 180 days old (+18%, $P < 0.05$). Offspring in the SL group showed higher visceral fat mass at 21 and 180 days old (+176 and +52%, respectively, $P < 0.05$), but plasma leptin was higher only at 21 days (+88%, $P < 0.05$). The SL offspring showed higher plasma TSH, 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) at 21 days (+60, +91 and +68%, respectively, $P < 0.05$), while the opposite was observed at 180 days regarding thyroid hormones (T_3 , -10%; and T_4 , -30%, $P < 0.05$), with no difference in TSH levels. In hypothalamus, no change was observed in the leptin signalling pathway at 21 days. However, lower janus tyrosine kinase 2 (JAK2) and phosphorilated-signal transducer and activator of transcription-3 (p-STAT3) content were detected in adulthood. In pituitary, the SL group presented higher leptin receptors (Ob-R), JAK2 and p-STAT3 content at 21 days and lower JAK2 and STAT3 content at 180 days old. In contrast, in thyroid, the Ob-R expression was lower in young SL rats, while the adult SL group presented higher Ob-R and JAK2 content. We showed that postnatal EO induces short- and long-term effects upon the hypothalamic–pituitary–thyroid axis. These changes may help to explain future development of metabolic and endocrine dysfunctions, such as metabolic syndrome and hypothyroidism.

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Abbreviations EO, early overnutrition; HPT axis, hypothalamic–pituitary–thyroid axis; IL-6, interleukin-6; JAK2, janus tyrosine kinase 2; NL, normal litter; Ob-R, leptin receptor; p-STAT3, phosphorilated-signal transducer and activator of transcription-3; SL, small litter; STAT3, signal transducer and activator of transcription 3; T_3 , 3,5,3'-triiodothyronine; T_4 , thyroxine; TNF- α , tumour necrosis factor- α ; TRH, thyrotrophin-releasing factor; TSH, thyroid-stimulating hormone.

It has been shown that adverse situations during lactation, such as malnutrition and hormonal changes, could permanently affect the nutritional and hormonal status of the progeny (Passos *et al.* 2004; Bonomo *et al.* 2007; Dutra *et al.* 2007; Fagundes *et al.* 2007; Trevenzoli, 2007). The

prevalence of childhood obesity is increasing worldwide at alarming rates (Hedley *et al.* 2004). Besides genetic factors, epigenetic environmental factors, such as dietary intake, can contribute to development of obesity. This association has been termed metabolic imprinting or programming,

which is defined as a biological phenomenon that determines the association between physical and chemical stimuli in early life and future functional status (Lucas, 1994; Barker, 2004; de Moura *et al.* 2008).

Overnutrition during early postnatal life represents a risk factor for persistent obesity and associated metabolic and cardiovascular disturbances. It has been shown that animals raised in small litters (SL) have an accelerated body weight gain before weaning, which is associated with permanent modulation of adiposity and hypothalamic circuits that control food intake and energy balance in adulthood (Faust *et al.* 1980; Plagemann *et al.* 1999*a,b*). Some experimental studies have reported that neonatal overfeeding causes persistent hyperphagia, obesity, elevated serum triacylglycerols, increased systolic blood pressure and hyperinsulinaemia in later life (You *et al.* 1990; Plagemann *et al.* 1992, 1999*c*; Paramore *et al.* 1999; Boullu-Ciocca *et al.* 2005; Davidowa & Plagemann, 2007; López *et al.* 2007; Rodrigues *et al.* 2007).

Leptin is a hormone secreted by adipose tissue, which reduces food intake and increases energy expenditure via specific hypothalamic signals, maintaining the body weight homeostasis (Pelleymounter *et al.* 1995; Schwartz *et al.* 1996; Friedman & Halaas, 1998). Leptin acts through the leptin receptors (Ob-R), which belong to the cytokine receptor class I superfamily. Five alternatively spliced isoforms of Ob-R (a, b, c, d and e) with different lengths of C-termini have been identified in mice (Lee *et al.* 1996). The long form (Ob-Rb) and the short form (Ob-Ra) are the most studied isoforms, and Ob-Rb is fully capable of activating intracellular signalling (Sahu, 2004). Leptin action mediated by leptin receptors involves multiple pathways, such as the janus tyrosine kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) pathway, which is the best characterized leptin signalling pathway. Leptin binding to the Ob-Rb initiates tyrosine phosphorylation by JAK2. Phosphorylated Ob-Rb recruits STAT3 proteins, which are activated through phosphorylation by JAK2 (Vaisse *et al.* 1996). The activated STAT3 proteins dimerize and translocate to the nucleus, stimulating gene transcription (Hekerman *et al.* 2005). The JAK2–STAT3 pathway stimulates transcription of suppressor of cytokine signalling 3 (SOCS3), a negative regulator of leptin signalling following Ob-Rb activation (Zhang *et al.* 1994; Vaisse *et al.* 1996).

It has been revealed that various peripheral organs have detectable levels of mRNA encoding the Ob-Rb, supporting the view that leptin has many peripheral actions, including suppression of insulin secretion, stimulation of cytokine production and control of development of reproductive organs (Muoio & Lynis Dohm, 2002). Leptin also has a well-known stimulatory role in thyroid function, mainly increasing thyrotrophin-releasing hormone (TRH) expression in the

hypothalamus (Legradi *et al.* 1997). The detection of Ob-R in human (Jin *et al.* 1999, Knerr *et al.* 2001) and rodent pituitary (Jin *et al.* 2000; Sone *et al.* 2001; Vicente *et al.* 2004) reinforces its direct action on this gland, suggesting that leptin may act as a regulator of thyroid-stimulating hormone (TSH) release. In addition, Nowak *et al.* (2002) showed that rat thyroids express the Ob-Rb and the gland function is stimulated by leptin in euthyroid non-fasted female rats. Other studies have shown that leptin regulates thyroid function, but the investigations of the possible effects of leptin in this tissue are rather limited and controversial (Orban *et al.* 1998; Pinkney *et al.* 1998; Isozaki *et al.* 2004; de Oliveira *et al.* 2007). Initially, leptin was viewed as a hormone designed to prevent obesity, but several studies now suggest that leptin signals the switch from fed to starved state (Baskin *et al.* 2000; Flier *et al.* 2000), adapting the organism to a more economic energy expenditure. Disturbance of thyroid function is associated with marked changes in both energy expenditure and body weight, and it seems that leptin and thyroid hormones play mutual roles.

In obesity hyperleptinaemia and leptin resistance have been reported; therefore, it is interesting also to evaluate the peripheral action of leptin, since it was already demonstrated a dissociate effect for the sympathetic nervous system (Mark *et al.* 2004). Here, we investigated the short- and long-term effects of postnatal early overnutrition (EO) on thyroid gland function, through the evaluation of plasma thyroid hormones and TSH. We also evaluated the plasma leptin levels and the proteins of the leptin signalling pathway in the hypothalamic–pituitary–thyroid (HPT) axis of young and adult animals raised in small litters.

Methods

Experimental model

Wistar rats were housed in a room maintained at 23–25°C with a 12 h–12 h light–dark cycle. Three-month-old, virgin female rats were caged with male rats at a ratio of 3:1. During pregnancy and lactation, mothers were housed in individual cages and were provided with water *ad libitum* and standard pellet diet (commercial control diets for rats). To induce postnatal early overnutrition (EO), 3 days after birth, the litter size was adjusted to three male rats per litter (small litter; SL group; Plagemann *et al.* 1992; Rodrigues *et al.* 2007). In control litters, the litter size was adjusted to 10 pups per litter (normal litter; NL group). After weaning (day 21), the SL and NL pups received a commercial diet. The number of animals analysed in our study was 12 per group per period studied from 16 different litters (8 SL litters and 8 NL litters). Body weight gain was measured throughout life. Food intake was measured by determination of 24 h consumption of

standard pellets from 30 to 180 days old. Both groups of offspring were killed when they were 21 or 180 days old, and we collected blood, hypothalamus, pituitary gland, thyroid gland and visceral fat mass. Animals were exsanguinated after anesthesia with pentobarbital. The use of the animals according our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEA/184/2007), which based their analysis on the principles described in the *Guide for the Care and Use of Laboratory Animals* (Bayne, 1996).

Body fat and protein mass

At the day of killing, visceral fat mass was excised (mesenteric, epididymal and retroperitoneal white adipose tissue) and immediately weighed for evaluation of central adiposity (Toste *et al.* 2006b).

Fat and protein mass were determined by carcass analysis as reported previously (Fagundes *et al.* 2007). After killing, the offspring were eviscerated, carcasses were weighed, autoclaved for 1 h and homogenized in distilled water (1:1 w/v). Samples of the homogenate were stored at 4°C for analysis.

Three grams of homogenate were used to determine fat mass gravimetrically. Samples were hydrolysed in a shaking water bath at 70°C for 2 h with 30% KOH and ethanol. Total fatty acids and free cholesterol were removed by three successive washings with petroleum ether. After drying overnight in a vacuum, all tubes were weighed and data expressed as grams of fat per 100 g carcass.

Protein mass was determined in 1 g homogenate. Tubes were centrifuged at 2000g for 10 min. Total protein concentrations were determined by the method of Lowry *et al.* (1951). Results were expressed as grams of protein per 100 g carcass.

Western blotting analysis

To obtain the cell extracts, the hypothalamus, pituitary and thyroid were homogenized in ice-cold lysis buffer (50 mM Hepes, pH 6.4, 1 mM MgCl₂, 10 mM EDTA and 1% Triton X-100) containing the following protease inhibitors: 10 µg/µl aprotinin, 10 µg/µl leupeptin, 2 µg/µl pepstatin and 1 mM phenylmethylsulphonic fluoride (Sigma-Aldrich, St Louis, MO, USA). The Ob-R, JAK2, STAT3 and p-STAT3 content was analysed by Western blotting, using actin as internal control.

Total protein content in the homogenate was determined (Bradford, 1976), and the cell lysates were denatured in sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and heated at 95°C for 5 min. Samples (50 µg total protein) were carried to 8, 10 or 12% SDS-PAGE, according to the molecular

weight of each protein, and transferred to polyvinylidene filters (PVDF Hybond-P, Amersham Pharmacia Biotech, New Jersey, NJ, USA). Rainbow markers (Amersham Biosciences, Uppsala, Sweden) were run in parallel to estimate molecular weights. Membranes were blocked with 5% non-fat milk in the Tween-Tris-buffered saline (Tween-TBS; 20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0.1% Tween-20). Primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) used were anti-Ob-R (1:5000); anti-JAK2 (1:500); anti-STAT3 (1:500); anti-p-STAT3 (1:500); and anti-actin (1:500). The PVDF filters were washed three times with Tween-TBS, followed by 1 h incubation with appropriate secondary antibody conjugated to biotin (Santa Cruz Biotechnology, Inc.). Then, filters were incubated with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA, USA). All Western blots were allowed to react with horseradish peroxidase substrate (ECL-plus; Amersham Pharmacia Biotech) and then exposed to X-ray film for between 10 s and 30 min. Images were scanned, and the bands were quantified by densitometry, using ImageJ 1.34s software (Wayne Rasband National Institute of Health, Boston, MA, USA).

Measurement of plasma hormones

Blood was obtained, just before sacrifice, by cardiac puncture and then quickly centrifuged (1000g, 4°C, 30 min) and plasma stored at -20°C until assayed. All measurements were performed in a unique assay. Plasma leptin concentration was determined by radioimmunoassay (RIA) kit (Linco Research, St Charles, MO, USA). Sensitivity limit and intra-assay variation were 0.5 ng ml⁻¹ and 6.9%, respectively. Plasma total 3,5,3'-triiodothyronine (T₃) and free thyroxine (T₄) were measured by RIA (ICN Pharmaceuticals, Inc., Los Angeles, CA, USA). Sensitivity limits were 25 ng dl⁻¹ for T₃ and 0.3 ng dl⁻¹ for T₄, and intra-assay variation was 3.6 and 7.5%, respectively. Plasma TSH was measured by specific RIA, using reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIH, Bethesda, MD, USA). Sensitivity limit was 0.18 ng ml⁻¹, and intra-assay variation was 6.1%.

Statistical analysis

Data are reported as means ± s.e.m. GraphPad Prism 4 program was used for statistical analyses and graphics. Two-way ANOVA and Bonferroni's test were used to analyse body weight and food intake evolutions. A non-parametric test (Mann-Whitney *U* test) was used for plasma TSH. The other experimental observations were analysed by Student's unpaired *t* test, with significance level set at *P* < 0.05.

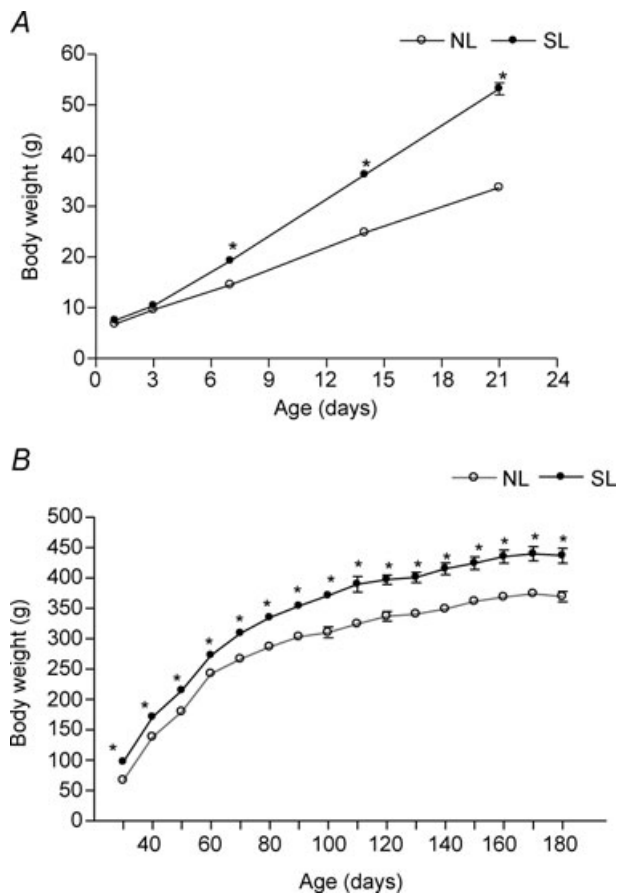


Figure 1. Postnatal EO induces early-onset obesity and overweight in adulthood

Body weight was evaluated in NL (●; $n = 12$) and SL groups (○; $n = 12$) during lactation (A) and after weaning (B). Results are expressed as means \pm s.e.m. * $P < 0.05$.

Results

Postnatal EO induces early-onset obesity, persistent hyperphagia and overweight in adult rats

Body weight evolution from birth until adulthood is shown in Fig. 1. Rat offspring raised in small litters had

a significantly higher body weight gain when they were 7 days old compared with control animals (+33%), 56% higher at 21 days old and 18% higher at 180 days old.

Figure 2 shows that SL rats presented higher daily relative food intake from weaning until adulthood (approximately 10%).

Postnatal EO alters the fat and lean mass in both young and adult rats

Total and visceral fat mass were higher in both young (+237 and +176%, respectively, $P < 0.05$; Table 1) and old animals (+38 and +52%, respectively, $P < 0.05$; Table 1). In contrast, postnatal EO induced an increase in protein mass in young rats, while a decrease was observed at adulthood (+41 and -14%, respectively, $P < 0.05$; Table 1).

Thyroid gland mass in young and adult NL and SL rats

The SL rats presented had greater absolute thyroid gland mass at both time points (+62% at 21 days and +21% at 180 days, $P < 0.05$, Fig. 3A); however, no change was detected between groups in the relative thyroid mass (Fig. 3B).

Plasma hormone concentrations

Animals raised in small litters had higher plasma leptin values when they were 21 days old (+88%, $P < 0.05$); however, no difference was observed when they were 180 days old. The SL offspring showed higher plasma T_3 and T_4 at weaning (+91 and +68%, respectively, $P < 0.05$), while the opposite profile was observed in adulthood (-10 and -30%, respectively, $P < 0.05$). Plasma TSH was higher (+62%, $P < 0.05$) in 21-day-old SL rats, and no difference was observed in adult rats (Table 1).

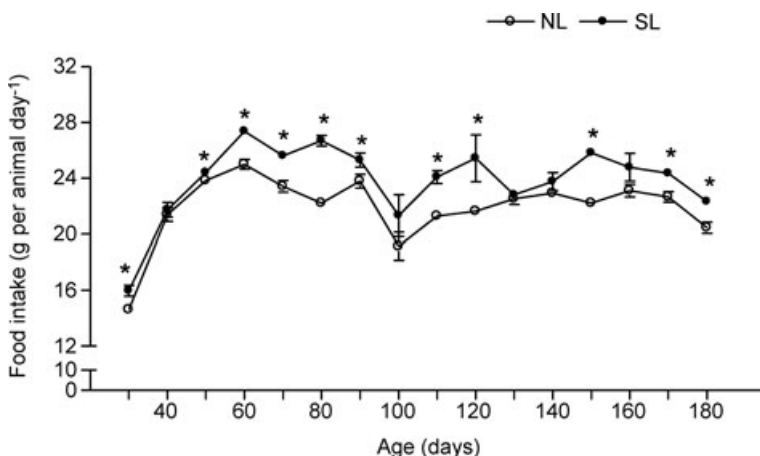


Figure 2. Postnatal EO induces persistent hyperphagia

Mean food intake was evaluated in NL (●; $n = 12$) and SL groups (○; $n = 12$) during development. Results are expressed as means \pm s.e.m. * $P < 0.05$.

Table 1. Body composition and plasma hormone levels of young and adult rats

	21 days		180 days	
	NL	SL	NL	SL
Total fat mass (%)	3.65 ± 0.27	12.32 ± 0.62*	7.57 ± 0.50	10.50 ± 1.09*
Visceral fat mass (%)	0.39 ± 0.05	1.08 ± 0.12*	2.01 ± 0.17	3.06 ± 0.41*
Protein mass (%)	5.82 ± 0.35	8.22 ± 0.45*	6.88 ± 0.28	5.93 ± 0.13*
Leptin (ng ml ⁻¹)	0.99 ± 0.23	1.88 ± 0.09*	0.99 ± 0.10	1.06 ± 0.08
TT ₃ (ng dl ⁻¹)	48.21 ± 2.93	92.36 ± 3.48*	53.07 ± 1.07	48.17 ± 2.07*
FT ₄ (ng dl ⁻¹)	0.94 ± 0.05	1.58 ± 0.14*	2.73 ± 0.15	1.92 ± 0.19*
TSH (ng ml ⁻¹)	0.78 ± 0.09	1.26 ± 0.09*	2.84 ± 0.37	2.76 ± 0.35

Results are expressed as means ± s.e.m. of 12 rats per group. * $P < 0.05$.

Leptin signalling pathway in the HPT axis

Figures 4 and 5 show the content of leptin signalling proteins (Ob-R, JAK2, STAT3 and p-STAT3) in the hypothalamus from NL and SL groups as assessed by Western blotting technique. At 21 days old, no change was detected in leptin signalling proteins (Fig. 4). However, 180-day-old SL animals had a lower hypothalamic JAK2 content (−59%, $P < 0.05$, Fig. 5A and C) and a decrease in STAT3 phosphorylation (−53%, $P < 0.05$, Fig. 5A and E) with no alteration in Ob-R and STAT3 expression (Fig. 5A, B and D).

In the pituitary gland, young SL animals had a higher Ob-R and JAK2 content (+134 and +55%, respectively, $P < 0.05$, Fig. 6A, B and C) and consequently higher STAT3 phosphorylation (+110%, $P < 0.05$, Fig. 6A and E). In adulthood, we observed an opposite profile, with lower JAK2 and STAT3 content (−52 and −20%, respectively, $P < 0.05$, Fig. 7A, C and D). However, no change was detected in STAT3 phosphorylation in pituitary from SL adult animals (Fig. 7A and E).

Regarding the leptin signalling proteins in the thyroid gland (Figs 8 and 9), Ob-R expression was lower in SL rats at 21 days old (−38%, $P < 0.05$, Fig. 8A and B), while at 180 days old, SL rats showed higher Ob-R and JAK2 contents (+40 and +60%, respectively $P < 0.05$, Fig. 9A, B and C) when compared with NL animals. No difference was observed in thyroid STAT3 and p-STAT3 content at either studied time point.

Discussion

In the present study, we observed that postnatal EO induced by small litter size causes a dramatic increase in body weight gain during lactation and programmes for overweight and persistent hyperphagia in later life. These findings are in accordance with earlier studies (Faust *et al.* 1980; Plagemann *et al.* 1992, 1997; Velkoska *et al.* 2005; Davidowa & Plagemann, 2007; Rodrigues *et al.* 2007). Interestingly, adult SL animals presented a downregulation of the leptin signalling pathway in the hypothalamus,

characterizing a central leptin resistance. Leptin resistance has been well established in obesity (Sahu, 2002, 2003; Sandoval & Davis, 2003) and could explain the persistent hyperphagia observed in animals raised in small litters. In this study, we also showed that rats overfed in early life presented higher central adiposity at 21 and 180 days. Moreover, lower protein mass observed in adulthood indicates that the increased body weight is mainly due to a higher body fat mass. The adipose tissue distribution in obese subjects is important because the regional fat

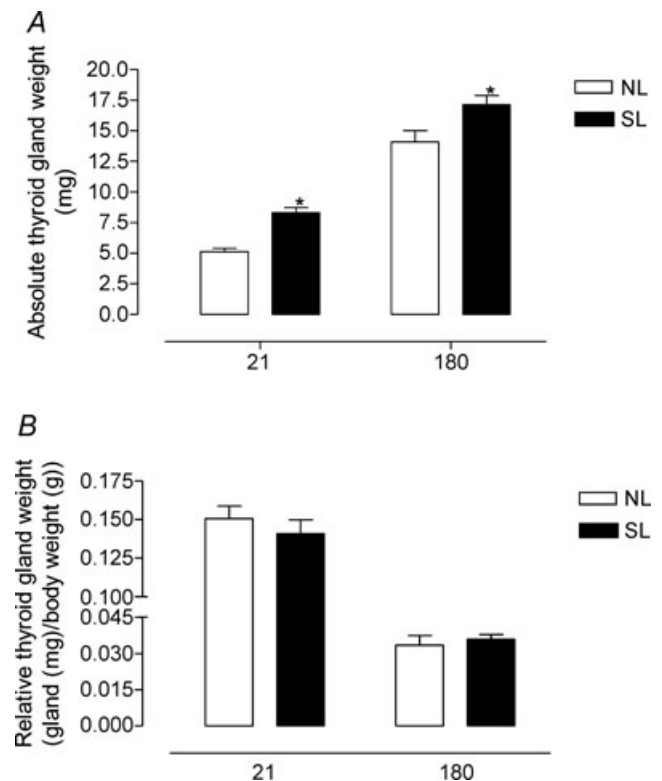


Figure 3. Effect of postnatal EO on thyroid gland weight of young and adult NL and SL rats

Thyroid weight was evaluated in NL (filled bars; $n = 12$) and SL groups (open bars; $n = 12$) at 21 and 180 days old. The figure shows the absolute (A) and relative thyroid weight (B). Results are expressed as means ± s.e.m. * $P < 0.05$.

deposition confers different levels of cardiovascular risk (Grassi *et al.* 2004; Velkoska *et al.* 2005; Hamdy *et al.* 2006; Mathieu *et al.* 2008). Therefore, our findings suggest that neonatal obesity could be a risk factor for cardiovascular disease in later life, considering the higher visceral adiposity.

There are scarce data concerning the programming effect in females. Some programming models showed a sex-specific effect (Moura *et al.* 2002; Lu *et al.* 2007; Darnaudéry & Maccari, 2008). In the EO model, we found one only paper, by Basset & Craig (1998), which discusses this gender effect. However, generally in programming experiments the utilization of both males and females in the same experiment could be a confounding factor; for example, males can ingest more milk than females.

Recently, Xiao *et al.* (2007) showed lower uncoupling protein 1 (UCP1) expression in brown adipose tissue (BAT) from adult rats raised in small litters. Impaired

BAT thermogenesis has been reported in genetic models of obesity (Commins *et al.* 1999; Masaki *et al.* 2000); conversely, mice overexpressing UCP1 are obesity resistant (Kopecky *et al.* 1995). Therefore, lower UCP1 in BAT reported previously in adult early overfed animals suggests reduced energy expenditure and consequently increased body weight in this experimental model (Xiao *et al.* 2007). However, those authors did not evaluate the thyroid function.

Corroborating previous studies, animals raised in small litters presented hyperleptinaemia at weaning (Plagemann *et al.* 1999c; Sanchez *et al.* 2003). However, no change was detected in plasma leptin of SL offspring at 180 days old. Two studies (Velkoska *et al.* 2005; López *et al.* 2007) showed that postnatal EO causes hyperleptinaemia until 60 days, and one of them (Velkoska *et al.* 2005) reported that this change was not observed in SL animals when they were 120 days old. To the best of our knowledge, there

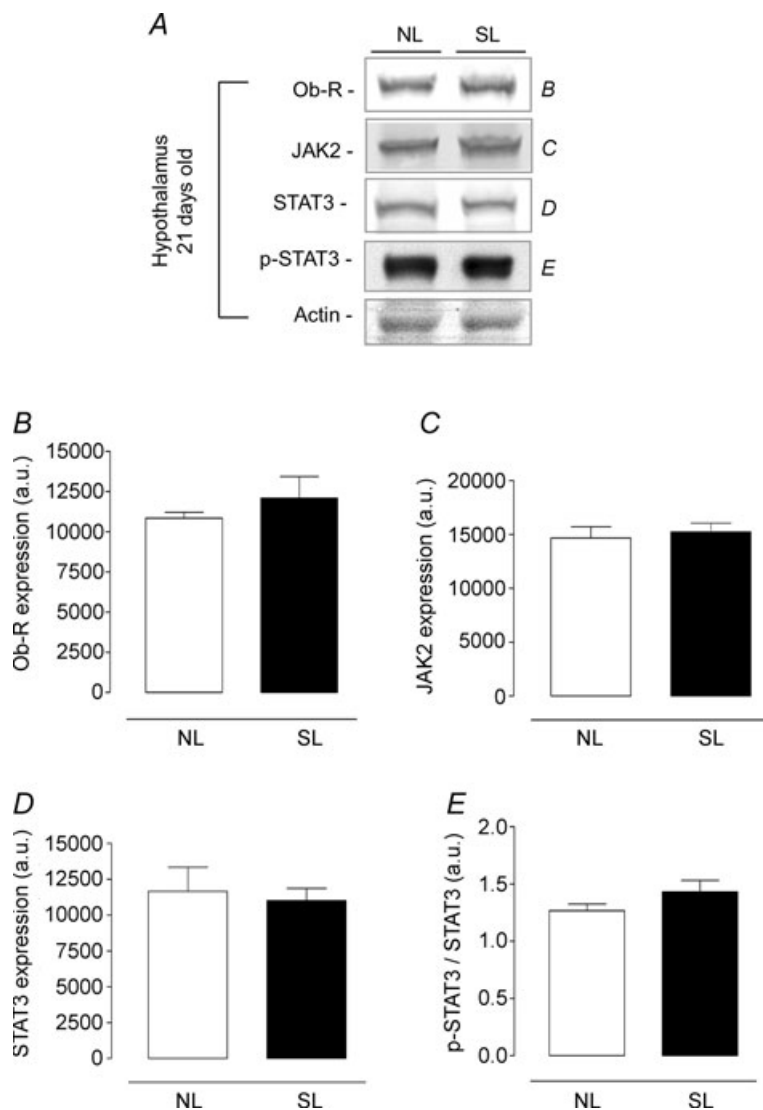


Figure 4. Effect of postnatal EO on expression of proteins of the leptin signalling pathway in the hypothalamus of young rats (21 days)

Homogenates of hypothalamus from SL and NL groups were obtained, detection of Ob-R (B; $n = 7$), JAK2 (C; $n = 7$), STAT3 (D; $n = 7$) and p-STAT3 (E; $n = 7$) was done by Western blotting. The bands in A represent the expression of proteins in hypothalamus homogenate from individual NL and SL animals. The Ob-R, JAK2, STAT3 and p-STAT3 contents were quantified by scanning densitometry of the bands (expressed in arbitrary units, a.u.). Actin was loaded as a control. Results are expressed as means + s.e.m. * $P < 0.05$. A representative experiment is shown from three independent experiments.

are no reports of leptin levels at 180 days old in animals raised in small litters. However, leptin is secreted mainly by the subcutaneous adipose tissue and here we showed an increase in only the visceral fat mass. Since most of the fat in adult rats is in the subcutaneous tissue, our data suggest that SL adult rats do not present increased subcutaneous fat mass when compared with control animals.

Thyroid gland can be regulated by nutritional status (Moura *et al.* 1987; Flier *et al.* 2000). Here we showed that nutritional changes in early life alter thyroid function during development. Animals raised in small litters presented higher plasma T_3 , T_4 and TSH at weaning, while the opposite profile was detected at 180 days. Interestingly, some studies reported the same profile of TH and TSH levels in obese children (Zheng *et al.* 1996; Kopelman, 2000; Stichel *et al.* 2000). In obese children from China, higher T_3 was positively correlated with body fat mass (Zheng *et al.* 1996). Similarly, other two studies showed a positive association between increased T_3 and TSH levels and the degree of overweight in childhood (Kiortsis

et al. 1999; Stichel *et al.* 2000). It has been reported that altered TSH and T_3 in obese children can be normalized after weight reduction (Kiortsis *et al.* 1999), reinforcing the view that obesity alters thyroid function, possibly through the action of leptin. Since leptin is well known to stimulate thyroid function, mainly increasing TRH expression (Legradi *et al.* 1997), we hypothesized that the higher TSH levels in young SL rats may be due to an increase in leptin action at the hypothalamus. This increase in thyroid function could be physiologically important to counterbalance the higher adiposity induced by early overfeeding.

An association between increased T_3 and TSH levels and excess body weight has also been reported in obese human adults (Bray *et al.* 1973, 1976). In the present study, adult animals raised in small litters were programmed for lower thyroid hormone levels, with normal TSH. In accordance with our data, some studies have also shown no association between TSH and body weight (Rosenbaum *et al.* 2000; Roti *et al.* 2000; Tagliaferri *et al.* 2001; Ritz *et al.* 2002). Probably, weight- or nutrition-related factors affect the

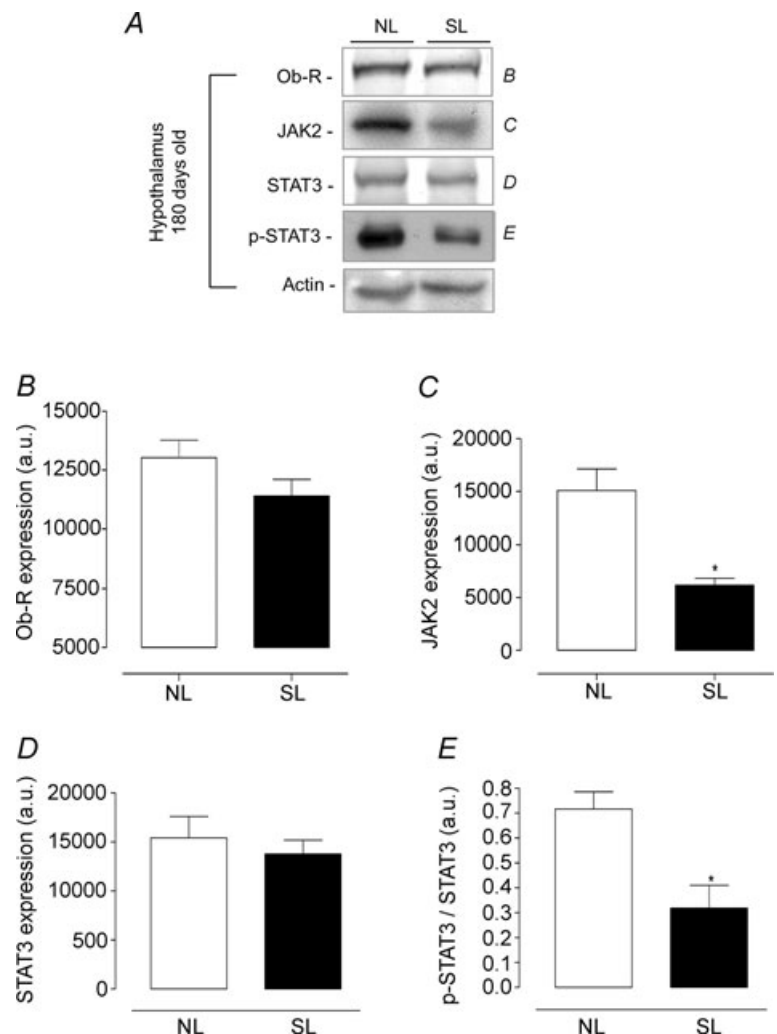


Figure 5. Effect of postnatal EO on expression of proteins of the leptin signalling pathway in in hypothalamus of adult rats (180 days)

Homogenates of hypothalamus from SL and NL groups were obtained, and detection of Ob-R (B; $n = 7$), JAK2 (C; $n = 7$), STAT3 (D; $n = 7$) and p-STAT3 (E; $n = 7$) was done by Western blotting. The bands in A represent the expression of proteins in hypothalamus homogenate from individual NL and SL animals. The Ob-R, JAK2, STAT3 and p-STAT3 contents were quantified by scanning densitometry of the bands. Actin was loaded as a control. Results are expressed as means \pm S.E.M. * $P < 0.05$. A representative experiment is shown from three independent experiments.

long-term regulation of the HPT axis, but the mechanisms involved are still unclear.

According to some authors (Dussault *et al.* 1982; Walker and Courtin, 1985; Pracyk *et al.* 1992; Wilcoxon & Redei, 2004; Moura *et al.* 2008), neonatal transient hyperthyroidism programmes for future hypothyroidism. In the adult animals, despite the lower thyroid hormone serum levels of SL rats, TSH was inappropriately normal. Thus, it is possible that, after weaning, an increase in thyroid hormone feedback could permanently inhibit the TRH–TSH axis, maybe through the increase in central deiodinase expression and/or activity of SL rats. Also the TSH level of these animals might be only immunologically normal but its bioactivity could be lower, in the case of lower TRH (Gesundheit *et al.* 1986).

Despite a lack of change in the expression of proteins of the leptin signalling pathway in hypothalamus at 21 days old, the hyperleptinaemia *per se* can increase TRH production, which consequently stimulates TSH secretion. In contrast, López *et al.* (2005) showed lower hypothalamic Ob-Rb mRNA expression in EO animals when they were 24 days old. However, in our study we showed the Ob-R protein content that included the Ob-Rb and other isoforms of the leptin receptor, while López *et al.* (2005) reported the expression of Ob-Rb mRNA. It is possible that the metabolism of this protein can also be altered and, despite the lower synthesis, a reduction in catabolism might have kept the content unchanged. However, although the antibody used in this study was not specific to the long form, it is unlikely that isoforms other

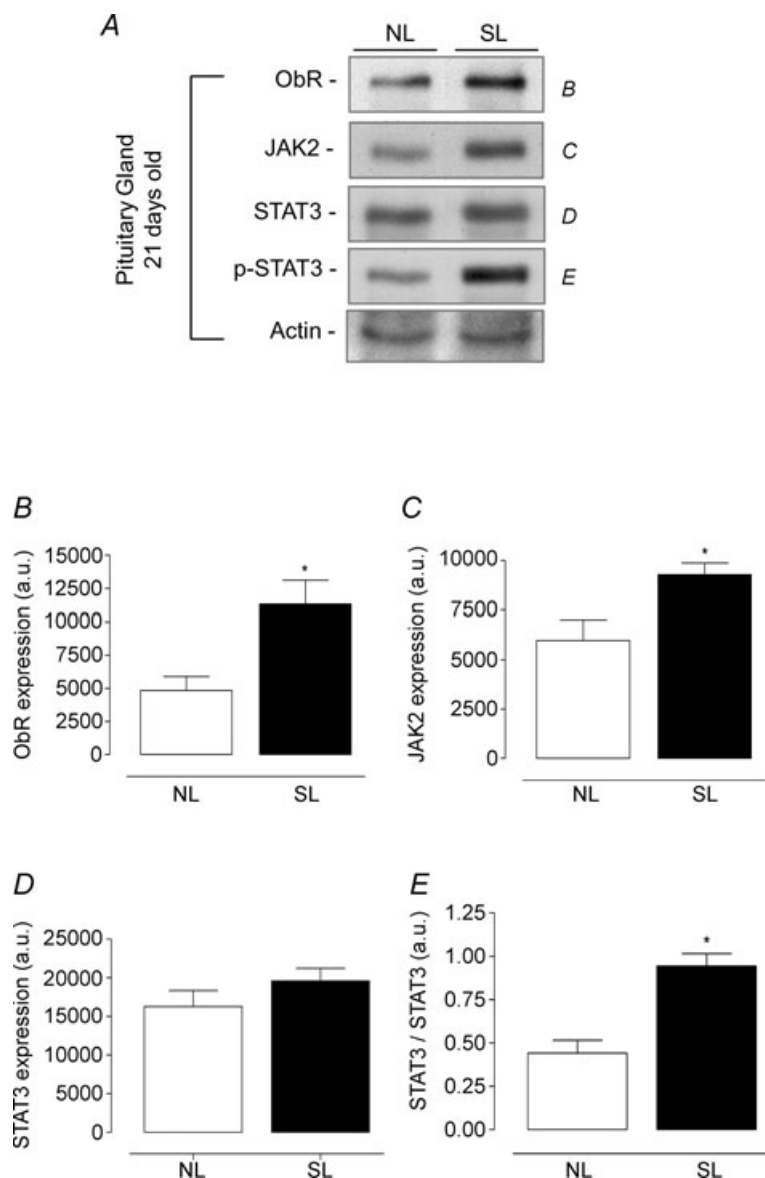


Figure 6. Effect of postnatal EO on expression of proteins of the leptin signalling pathway in pituitary of young rats (21 days)

Homogenates of pituitary from SL and NL groups were obtained, and detection of Ob-R (B; $n = 6$), JAK2 (C; $n = 6$), STAT3 (D; $n = 6$) and p-STAT3 (E; $n = 6$) was done by Western blotting. The bands in A represent the expression of proteins in pituitary homogenate from individual NL and SL animals. The Ob-R, JAK2, STAT3 and p-STAT3 contents were quantified by scanning densitometry of the bands. Actin was loaded as a control. Results are expressed as means + s.e.m. * $P < 0.05$. A representative experiment is shown from three independent experiments.

than Ob-Rb are present in hypothalamus in significant amounts. Thus, although the results of López *et al.* (2005) could explain the apparent leptin resistance at this age, probably it is not related to the leptin signalling pathway and could be related to other mechanisms, such as leptin transport through the blood–brain barrier or influences over the peptides regulated by leptin. Moreover, until now, there have been no reports about leptin signalling proteins in hypothalamus from young animals submitted to postnatal EO.

In other models of neonatal programming, our group showed that leptin injections on the first 10 days of life programmed for hyperleptinaemia and hypothyroidism at 30 days old, as well as for hyperleptinaemia and hyperthyroidism at 150 days old (Toste *et al.* 2006*a,b*). In addition, these offspring when adult presented lower expression of Ob-Rb in the hypothalamus (Toste *et al.* 2006*b*). In our study, we did not show lower hypothalamic Ob-R expression; however, the JAK2 content and STAT3 phosphorylation were reduced, also indicating downregulation of the central leptin action. In earlier

studies, we also showed an association between maternal malnutrition during lactation and higher serum thyroid hormone levels in adulthood (Passos *et al.* 2002; Lisboa *et al.* 2008). In addition, the malnutrition of the offspring induced by blockage of the maternal serum prolactin at the end of lactation is related to hypothyroidism in adult life (Bonomo *et al.* 2008). Taken together, these data suggest an association between neonatal nutritional and hormonal status and thyroid function in adult life. In the two models mentioned above, leptin was increased in the neonatal period (Teixeira *et al.* 2002; Bonomo *et al.* 2005).

Initially, leptin was viewed as a hormone designed to prevent obesity, but nowadays this concept has been extended because leptin regulates many other systems, including the immune, reproductive and thyroid systems (Karlsson *et al.* 1997; Jin *et al.* 1999; Liu *et al.* 2007). It has been shown that leptin, *in vivo*, regulates the pituitary gland function, directly stimulating TSH production (Ahima *et al.* 1996; Seoane *et al.* 2000; Ortiga-Carvalho *et al.* 2002). Ortiga-Carvalho *et al.* (2002) showed that acute leptin injection in rats increased serum TSH

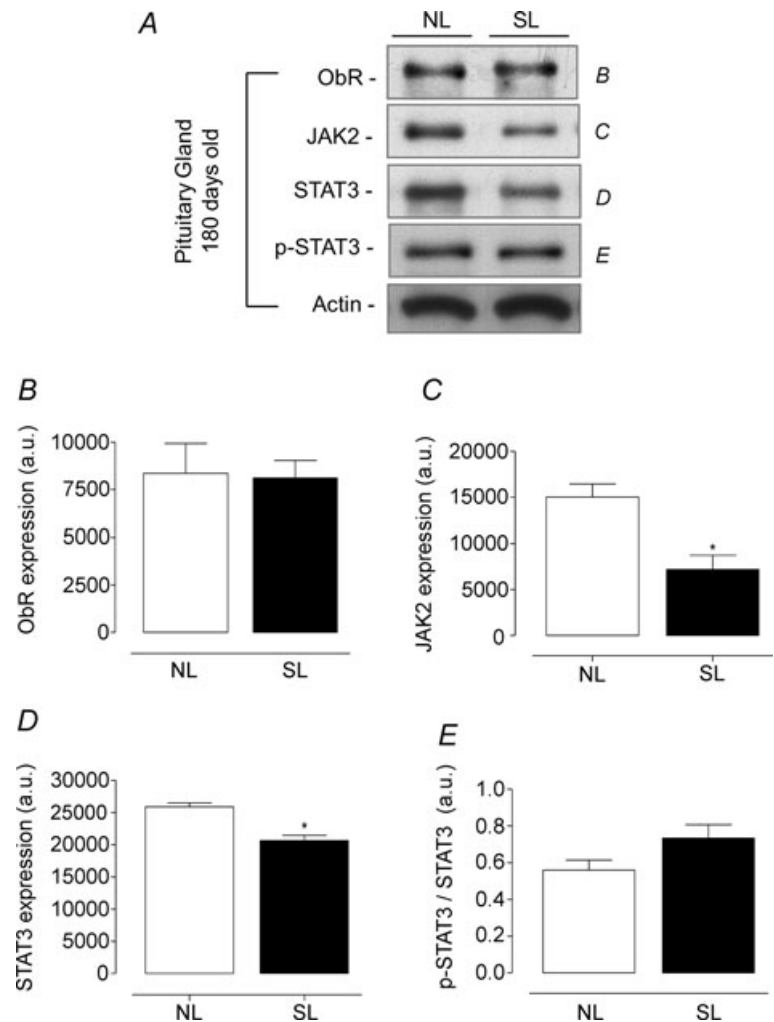


Figure 7. Effect of postnatal EO on expression of proteins of the leptin signalling pathway in pituitary of adult rats (180 days)

Homogenates of pituitary from SL and NL groups were obtained, and detection of Ob-R (B; *n* = 7), JAK2 (C; *n* = 7), STAT3 (D; *n* = 7) and p-STAT3 (E; *n* = 7) was done by Western blotting. The bands in A represent the expression of proteins in pituitary homogenate from individual NL and SL animals. The Ob-R, JAK2, STAT3 and p-STAT3 content were quantified by scanning densitometry of the bands. Actin was loaded as a control. Results are expressed as means + S.E.M.

**P* < 0.05. A representative experiment is shown from three independent experiments.

levels. In their study, leptin-incubated pituitary glands showed a dose-dependent decrease in TSH release, and the presence of antiserum against leptin resulted in an approximately 40% increase in TSH release. Several studies have demonstrated the presence of Ob-R in pituitary glands from humans (Jin *et al.* 1999; Knerr *et al.* 2001) and rats (Jin *et al.* 2000; Sone *et al.* 2001; Vicente *et al.* 2004), suggesting that leptin has direct effect in pituitary gland to regulate TSH secretion. Therefore, we investigated the content of leptin signalling proteins in pituitary gland from neonatal overfed animals.

At 21 days old, we observed an overexpression of Ob-R and JAK2 in pituitary gland, followed by a higher basal phosphorylation of STAT3 protein. Since young SL animals also showed higher serum leptin and TSH levels, we suggest that leptin could play a direct stimulatory effect upon TSH secretion, higher than in the control group. We observed an opposite profile in adult SL animals, which showed lower JAK2 and STAT3 expression. However, the lower JAK2 and STAT3 content did not alter the basal p-STAT3. Interesting, adult SL rats presented the same profile of leptin signalling pathway in the hypothalamus.

We observed a decrease in JAK2 and p-STAT3 content in hypothalamus from adult rats submitted to postnatal EO. Curiously, when adult, the SL animals showed no change in TSH and leptin levels, suggesting that other regulatory factors explain the leptin signalling changes in adulthood. We hypothesized that the lower expression of leptin signalling proteins in hypothalamus and pituitary gland could interfere in the negative feedback of thyroid hormones, which could explain the decrease in thyroid hormone levels without a change in plasma TSH levels in the adult SL group. In addition, it is possible that adult animals submitted to postnatal EO showed either an increase in pituitary sensitivity to thyroid hormones or a decrease in TSH bioactivity.

Several cytokines are produced by adipocytes (Lumeng *et al.* 2007; Zeyda & Stulnig, 2007; Heilbronn & Campbell, 2008). Interesting, animals raised in small litters have been shown to have a high content of tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in mesenteric adipose tissue at 150 days old (Boullu-Ciocca *et al.* 2008). Tumour necrosis factor- α has previously been shown to induce formation of reactive oxygen species in a variety

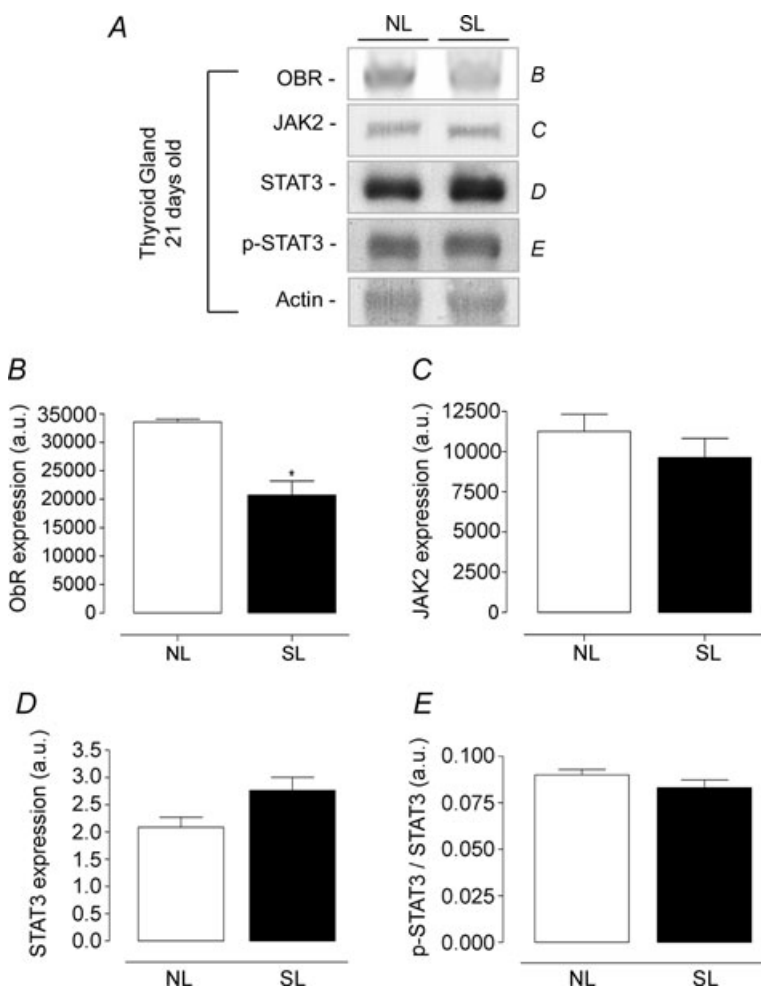


Figure 8. Effect of postnatal EO on expression of proteins of the leptin signalling pathway in thyroid of young rats (21 days)

Homogenates of thyroid from SL and NL groups were obtained, and detection of Ob-R (B; $n = 6$), JAK2 (C; $n = 6$), STAT3 (D; $n = 6$) and p-STAT3 (E; $n = 6$) was done by Western blotting. The bands in A represent the expression of proteins in thyroid homogenate from individual NL and SL animals. The Ob-R, JAK2, STAT3 and p-STAT3 contents were quantified by scanning densitometry of the bands. Actin was loaded as a control. Results are expressed as means + S.E.M. * $P < 0.05$. A representative experiment is shown from three independent experiments.

of tissues (Goossens *et al.* 1995; Javesghani *et al.* 2003). Reactive oxygen species are known to activate nuclear factor- κ B (Mercurio & Manning, 1999), which could regulate the expression of leptin signalling proteins in the hypothalamus and pituitary. Moreover, IL-6 and TNF- α have been reported to decrease HPT axis activity and TSH secretion (Torpy *et al.* 1998; Mönig *et al.* 1999). Based on these findings, we suggest that TNF- α and IL-6 could be other factors involved in the regulation of the leptin signalling pathway in hypothalamus and pituitary from SL animals.

Thyroid dysfunctions are associated with marked changes in body weight and energy expenditure, and it seems that both thyroid hormones and leptin could play a synergistic role. Several animal and human studies have investigated the association between leptin, thyroid hormones and thyroid dysfunction (Legradi *et al.* 1997; Seoane *et al.* 2000; Nowak *et al.* 2002; Ortiga-Carvalho *et al.* 2002; Zimmermann-Belsing *et al.* 2003; Isozaki *et al.* 2004; de Oliveira *et al.* 2007; Fagundes *et al.* 2007). In rodents, but not in humans, leptin receptors have been identified on thyroid cells (Nowak *et al.* 2002).

The function of leptin in peripheral tissues has yet to be determined, but it has been suggested that systemic leptin administration in rats stimulates thyroid gland growth and secretion thyroid hormones by mechanisms involving Ob-R (Nowak *et al.* 2002). Accordingly, EO animals showed higher absolute thyroid weight and changes in thyroid function when both young and adult. However, when we correlated the thyroid weight with the body weight (relative thyroid weight), no difference was observed between the groups. Thus, the thyroid gland mass is higher in SL offspring, but this increase is proportional to the higher body mass observed in these animals.

In this study, we demonstrated for the first time the content of proteins of the leptin signalling pathway in the thyroid gland. At 21 days old, SL offspring showed lower Ob-R expression in thyroid. Curiously, once again we observed an opposite profile in these animals when they were 180 days old, when there was an overexpression of thyroid Ob-R and JAK2 protein content. This inverse association between thyroid hormone levels and the content of some proteins in the leptin signalling pathway

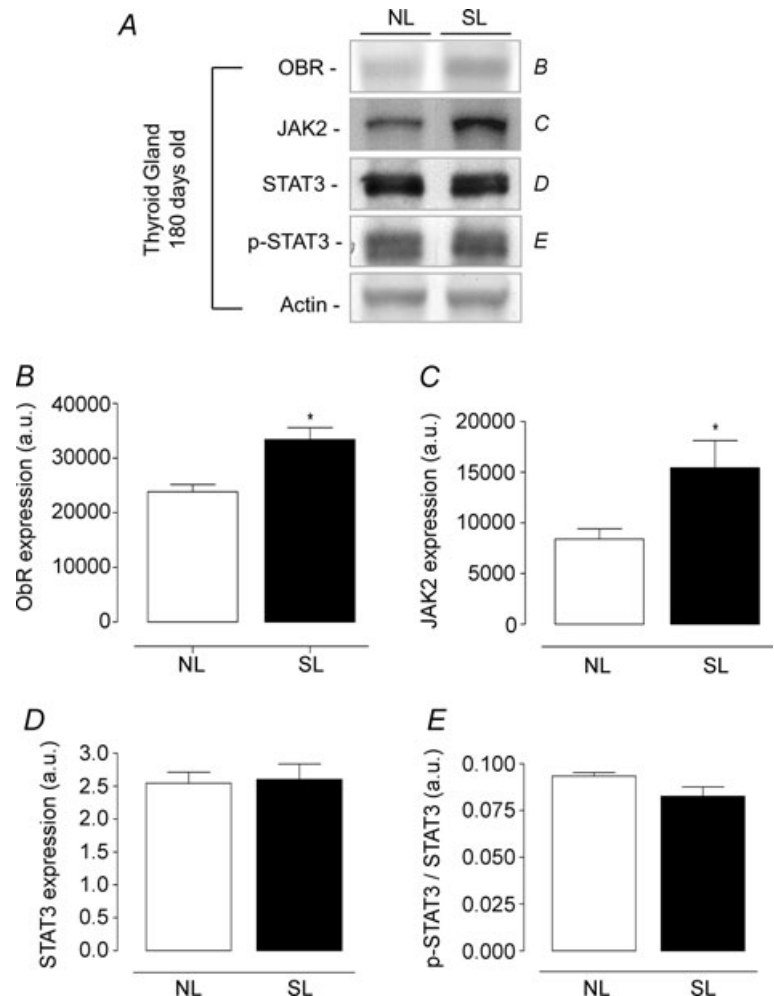


Figure 9. Effect of postnatal EO on expression of proteins of the leptin signalling pathway in thyroid of adult rats (180 days)

Homogenates of thyroid from SL and NL groups were obtained, and detection of Ob-R (B; $n = 7$), JAK2 (C; $n = 7$), STAT3 (D; $n = 7$) and p-STAT3 (E; $n = 7$) was done by Western blotting. The bands in A represent the expression of proteins in thyroid homogenate from individual NL and SL animals. The Ob-R, JAK2, STAT3 and p-STAT3 contents were quantified by scanning densitometry of the bands. Actin was loaded as a control. Results are expressed as means \pm S.E.M.

* $P < 0.05$. A representative experiment is shown from three independent experiments.

at both time points studied is remarkable. Interestingly, this profile is opposite to that observed in pituitary and thyroid glands. Few studies have shown the expression of thyroid Ob-R (Nowak *et al.* 2002; Isozaki *et al.* 2004), and little is known about the regulation of leptin receptors in thyroid gland. Nevertheless, in animals submitted to postnatal EO there are some putative factors, known to be altered in obesity, which may regulate the leptin signalling pathway at the pituitary and/or thyroid level. For example, TNF- α has been reported to inhibit thyroid specific gene expression, such as the Na⁺-I⁻ symporter (Pekary & Hershman, 1998) and TSH-induced hydrogen peroxide production (Kimura *et al.* 1997). Thus, it is possible that some factors, such as TNF- α and IL-6, could also interfere with leptin signalling in thyroid cells. Also, TSH, thyroid hormones or leptin could play a role in this process.

Hyperleptinaemia at 21 days could downregulate thyroid Ob-R expression, and higher TSH could have the same effect at this time. However, at 180 days, changes in the leptin signalling pathway could not be explained by TSH or leptin, because their levels were unchanged. Thus, it is possible that thyroid hormones could directly or indirectly regulate the leptin pathway in thyroid, suppressing this pathway at 21 days and failing to suppress it at 180 days. Studies with different models of hyper- and hypothyroidism, using antithyroid drugs, TSH or TRH injection, are necessary to elucidate the role of thyroid hormones in the leptin signalling pathway in thyroid gland.

In general terms, epigenetic mechanisms, such as DNA methylation or histone acetylation–deacetylation, induced by neonatal nutritional, hormonal or environmental factors, may lead to an increased risk of metabolic disorders in the adult offspring (de Moura *et al.* 2008). Thus, this explanation may help in understanding the mechanism involved in the permanent changes of thyroid function induced by overnutrition during lactation. Since this alteration might make overfed children more susceptible to thyroid disorders in adult life, it deserves epidemiological and prospective studies.

In summary, postnatal EO induces short- and long-term effects upon thyroid function that can directly or indirectly modulate the leptin signalling pathway in the rat HPT axis. Since changes in the leptin signalling pathway seem to be similar in hypothalamus and pituitary gland, and in the opposite direction in thyroid gland, we suggest that different regulatory factors are responsible for these differences in our programming model.

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