Effects of maternal nicotine exposure on thyroid hormone metabolism and function in adult rat progeny

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Abstract

Postnatal nicotine exposure leads to obesity and hypothyroidism in adulthood. We studied the effects of maternal nicotine exposure during lactation on thyroid hormone (TH) metabolism and function in adult offspring. Lactating rats received implants of osmotic minipumps releasing nicotine (NIC, 6 mg/kg per day s.c.) or saline (control) from postnatal days 2 to 16. Offspring were killed at 180 days. We measured types 1 and 2 deiodinase activity and mRNA, mitochondrial α-glycerol-3-phosphate dehydrogenase (mGPD) activity, TH receptor (TR), uncoupling protein 1 (UCP1), hypothalamic TRH, pituitary TSH, and *in vitro* TRH-stimulated TSH secretion. Expression of deiodinase mRNAs followed the same profile as that of the enzymatic activity. NIC exposure caused lower 5′-D1 and mGPD activities; lower TRβ1 content in liver as well as lower 5′-D1 activity in muscle; and higher 5′-D2 activity in brown adipose tissue (BAT), heart, and testis, which are in accordance with hypothyroidism. Although deiodinase activities were not changed in the hypothalamus, pituitary, and thyroid of NIC offspring, UCP1 expression was lower in BAT. Levels of both TRH and TSH were lower in offspring exposed to NIC, which presented higher basal *in vitro* TSH secretion, which was not increased in response to TRH. Thus, the hypothyroidism in NIC offspring at adulthood was caused, in part, by *in vivo* TRH–TSH suppression and lower sensitivity to TRH. Despite the hypothyroid status of peripheral tissues, these animals seem to develop an adaptive mechanism to preserve thyroxine to triiodothyronine conversion in central tissues.

Key Words
- lactation
- programing
- nicotine
- deiodinase
- thyroid hormones
- TSH
- α-glycerol-3-phosphate dehydrogenase
- TH receptor

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Introduction

Hormonal, nutritional, and environmental changes during critical periods of development (e.g., gestation and the postnatal period before weaning) can permanently change physiological parameters at adulthood. This process, known as metabolic programing, is associated with the development of several chronic diseases such as obesity, dyslipidemia, diabetes, and cardiovascular disease (Barker 2003, de Moura *et al.* 2008). It is already known that the programing phenomenon is based on epigenetic alterations (DNA methylation and histone acetylation)

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that change the pattern of expression of several genes. We have shown that the preweaning period is an important period for the action of several imprinting factors. In this sense, we have shown the programing of body composition and endocrine function in rodents by nutritional (Passos *et al*. 2002, Dutra *et al*. 2003, Fagundes *et al*. 2007, 2009, Lisboa *et al*. 2008, Rodrigues *et al*. 2009), hormonal (Gao *et al*. 2005, Toste *et al*. 2006*a*,*b*, Bonomo *et al*. 2008, Moura *et al*. 2008, de Moura *et al*. 2009) and environmental factors (Oliveira *et al*. 2009, Santos-Silva *et al*. 2011) during lactation.

Thyroid dysfunction is associated with important alterations in both energy expenditure and body weight (Pontikides & Krassas 2007), and the prevalence of obesity in children, adolescents, and adults is increasing worldwide at alarming rates (Hedley *et al*. 2004). Besides genetic factors, epigenetic environmental factors, such as tobacco smoke, can contribute to the development of obesity. Results from epidemiological studies indicate that maternal smoking during pregnancy might be a risk factor for obesity and hypertension in children and teenagers (Von Kries *et al*. 2002, Wideroe *et al*. 2003, Gao *et al*. 2005, Hill *et al*. 2005, Goldani *et al*. 2007). Furthermore, maternal smoking affects the thyroid function of infants (Karakaya *et al*. 1987, Chanoine *et al*. 1991, Gasparoni *et al*. 1998, Laurberg *et al*. 2004). Our group has obtained evidence that maternal exposure of rats to nicotine from the 2nd to the 16th day of lactation causes higher body fat, lower serum thyroxine (T_4) and triiodothyronine (T_3) with higher thyrotropin (TSH), featuring a primary thyroid hypofunction, as well as higher serum leptin in the suckling pups, at the end of nicotine exposure. Interestingly, these animals show a very quick recovery at weaning, because these parameters were normal. In adulthood, they were programed for overweight, secondary hypothyroidism (lower TSH, T_4, and T_3 levels), and hyperleptinemia (Oliveira *et al*. 2009). We also showed leptin and insulin resistance (de Oliveira *et al*. 2010), increased medullary adrenal function and serum glucocorticoid levels with higher levels of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) in adult offspring whose mothers were nicotine-exposed (Pinheiro *et al*. 2011).

Thyroid hormones (THs), T_4, and T_3 levels have well-known effects on growth and development, thermogenesis, and intermediary metabolism. Two enzyme systems are considered to be markers of thyroid function and reflect the action of TH: 5′-iodothyronine deiodinases and mitochondrial α-glycerol-3-phosphate dehydrogenase (mGPD). Deiodinases are responsible for the conversion of T_4 to T_3, the bioactive form of TH. Based on functional criteria and tissue distribution, deiodinases are classified as type 1 (D1) and type 2 (D2). In rats, D1 is predominantly found in liver, kidney, and thyroid, which are the main source of serum T_3. D2 is predominantly expressed in brain, pituitary, and brown adipose tissue (BAT), where it catalyzes the local conversion of T_4 to T_3. But according to some authors, both enzymes (D1 and D2) contribute similarly to the generation of plasma T_3 (Bianco *et al*. 2002, Bianco & Kim 2006, St Germain *et al*. 2009). mGPD, a TH status marker, controls the glycerol phosphate metabolism for gluconeogenesis or energy production and in cold-induced thermogenesis (Koza *et al*. 1996). The mGPD is located in the mitochondrial inner membrane, where it catalyzes irreversible oxidation reactions. In peripheral tissues, both D1 and mGPD are stimulated by circulating TH, whereas D2 is inhibited. Thus, a decrease in D1 and mGPD activities occurs in hypothyroidism but an increase in D2 activity in hyperthyroidism was, in contrast, observed to have the opposite effect upon these TH-dependent enzymes (Müller & Seitz 1994, St Germain *et al*. 2009). Most of actions of TH occur through the nuclear TH receptors (TR), which are encoded by two distinct genes, TRα and TRβ, located on mouse chromosomes 11 and 14. The molecular mechanism of TH action involves TR binding to TH responsive elements (TRE) on the promoter region of target genes and the recruitment of transcriptional co-factors (activators or repressors). At least, three isoforms (TRα1, TRβ1, and TRβ2) bind TH and display distinct pattern of expression among tissues and at different developmental stages (Yen 2001). How thyroid status affects the expression of these TR isoforms is controversial.

Mothers who stopped smoking during gestation, usually have a smoking relapse during lactation (McBride & Pirie 1990). We previously detected a programing for late secondary hypothyroidism in adult progeny in an experimental model of maternal exposure to nicotine only during the lactation period (Oliveira *et al*. 2009). As the decrease in THs and TSH is consistent but moderate (around 30% from normal), it would be interesting to examine the action of TH both peripherally and centrally. Thus, the goal of the present study was, besides that of enhancing our understanding about the thyroid dysfunction observed in adult offspring of dams that received nicotine during lactation, to assess the degree of hypothyroidism. We proposed the hypothesis that the alterations in TH metabolism and action could be responsible for a lower metabolic rate in this programing model, which could explain, at least in part, why the offspring of dams exposed to nicotine develop higher adiposity. Therefore, we evaluated the metabolism, action, and function of TH by measuring the deiodinase activities and mRNA levels in several tissues such as

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hypothalamus, pituitary, thyroid, liver, BAT, skeletal muscle, heart, and testis; mGPD activity and TRβ1 protein expression in liver; and uncoupling protein 1 (UCP1) expression in BAT. In addition, we also studied both TSH-releasing hormone (TRH) and TSH immunostaining, the *in vivo* TSH content of the pituitary gland, and *in vitro* TSH secretion after TRH stimulation in adult rats that had been nicotine-exposed in postnatal life. To our knowledge, no study has been specifically designed to analyze the consequences of maternal nicotine exposure on the TH-dependent proteins of the adult progeny.

Materials and methods

Wistar rats used in this study were kept in a temperature-controlled room (25±1 °C) with an artificial 0700 h light:1900 h darkness cycle. Female rats (3 months of age) were caged with male rats at the proportion of 3:1. After mating, each female rat was placed in an individual cage with free access to water and food until parturition. Our experimental model has been previously approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/189/2007 and CEUA 015/2009), which based its analysis on the principles adopted and promulgated by Brazilian Law no. 11.794/2008 (Marques *et al.* 2009). The experiments were carried on to minimize the number of animals used and the suffering caused by the procedures, following the ethical doctrine of the three 'Rs' – reduction, refinement, and replacement.

Model of postnatal nicotine exposure

On postnatal day 2 (PN2), 12 lactating rats were randomly divided into the following groups:

Nicotine group (NIC, *n*=6) Each dam was lightly anesthetized with thiopental, a 3×6 cm area on the back was shaved and an incision was made to permit s.c. insertion of an osmotic minipump (2ML2: Alzet, Cupertino, CA, USA). The use of minipumps allowed for a constant and controlled level of exposure throughout the lactation period. Pump implantation occurred on PN2 because pumps must be filled with the solution of interest and immersed in saline for 24 h before implantation to stabilize nicotine release. The pumps were prepared with nicotine-free base diluted in a saline solution (NaCl 0.9%) and set to deliver an initial dose of 6 mg/kg of nicotine per day for 14 days of lactation. Cotinine milk and serum concentrations had already been determined in this experimental model (de Oliveira *et al.* 2010, Santos-Silva *et al.* 2010). The chosen dose in our study produces plasma nicotine levels of approximately 25 ng/ml, which are similar to those observed in typical smokers (Lichtensteiger *et al.* 1988).

Control group (C, *n*=6) Pump implantation followed the procedures described above. However, pumps contained only saline solution.

Only dams with a litter size of ten offspring were used in order to avoid the influence of litter size on the programing effect. At birth, litter adjustment was performed and six male pups were kept per NIC or C mother to maximize lactation performance. We used two offspring randomly assigned from each mother (12 rats/group). The offspring were killed on PN180 by quick decapitation without prior anesthesia, and liver, BAT, hypothalamus, pituitary, thyroid, soleus (skeletal muscle), heart, and testis were collected. The tissues were dissected out, kept in liquid nitrogen, and immediately processed for enzymatic activity. For real-time PCR analysis, tissues were quickly frozen (−80 °C). Blood was collected, centrifuged to obtain serum (2000 ***g***, 20 min, 4 °C), and kept at −20 °C until assay.

Serum hormone levels

Serum free T_3 and T_4 levels were determined by RIA, using a commercial kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). The intra-assay variations were 2.9% (T_4) and 3.5% (T_3), with 0.045 ng/dl (T_4) and 0.06 pg/ml (T_3) as the limit of detection. Serum TSH was measured by specific RIA, using a rat TSH kit supplied by the National Institute of Health (Bethesda, MD, USA), and expressed in terms of the reference preparation provided (RP-3). The intra-assay variation was 2.3%, with 0.18 ng/ml as the lower limit of detection.

Determination of mGPD activity

Liver mGPD activity was measured using phenazine methosulfate (PMS) as an electron transporter between the reduced enzyme and iodonitrotetrazolium chloride violet (INT) (Oliveira *et al.* 2007). The assay was performed in the presence of 0.1 M DL-α-glycerophosphate diluted in KCN/KPB and a solution of 7.9 mM INT – 0.12 mM PMS. The samples were analyzed at 500 nm and values were expressed as absorbance (OD)/mg of mitochondrial protein.

Determination of 5′-iodothyronine deiodinases (D1 and D2) activities

5′-D1 and 5′-D2 activities were measured based on methods described previously (Pazos-Moura *et al.* 1991, Bates *et al.*

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1999, Lisboa *et al.* 2003*a*,*b*, 2008) by the release of ^{125}I from ^{125}I-reverse T_3 (rT_3) in liver, BAT, hypothalamus, pituitary, thyroid, soleus, heart, and testis homogenates.

Assays were carried out using phosphate buffer containing 1 mM EDTA, pH.6.9. The 5′-D1 assay was carried out in the presence of 1.5 μM rT_3, 10 mM dithiothreitol (DTT), and 100 nM T_4 (to inhibit 5′-D2). The 5′-D2 assay was performed with 2 nM rT_3, 40 mM DTT, and 1 mM propylthiouracil (PTU) (to inhibit 5′-D1). Equal aliquots of ^{125}I-rT_3 (27.8 MBq/μg – PerkinElmer/NEN, Boston, MA, USA) were purified by paper electrophoresis and placed into each assay tube. The reaction was started by addition of the following amount of protein (μg) samples: 35–270 liver, 38–135 BAT, 427–432 hypothalamus, 210–245 pituitary, 217–257 thyroid, 170–212 soleus, 246–338 heart, and 220–255 testis. A blank tube containing 50 μl of the substrate solution and 50 μl of buffer was run in parallel with each assay, and had its values subtracted from enzyme samples. The reactions were performed in a shaking-bath at 37 °C, and stopped after 30 min (liver and thyroid 5′-D1), 60 min (hypothalamic, pituitary, and thyroid 5′-D2), or 120 min (muscle 5′-D1, BAT, heart, and testis 5′-D2) by addition of a mixture of 8% BSA and 10 mM PTU, followed by 20% cold trichloroacetic acid. The samples were centrifuged (1500 ***g***, 4 °C, 5 min) and 200 μl of the supernatants were applied to Dowex 50 W-X2 columns (100–200 mesh hydrogen from Bio-Rad). Free ^{125}I, eluted from the column with 10% acetic acid, was measured using a gamma-counter. The percentage deiodinization in the presence of the enzyme was around 10–20%. The amount of free ^{125}I in the blank was generally $<$1–2% of the total radioactivity in the reaction mixture. The specific enzyme activity was expressed in fentomoles, picomoles, or nanomoles of rT_3 deiodinated per hour milligram protein.

Determination of *Dio1* and *Dio2* mRNA levels

Total RNA was extracted using a standard method (TRIzol Reagent, Life Technologies, Carlsbad, CA, USA). RT and PCR analyses were carried out on 1 μg of total RNA from liver, soleus, BAT, heart, and testis using a Superscript III Kit (Invitrogen). Real-time RT-PCR analyses were performed in a fluorescent temperature cycler (Applied Biosystems 7500, Life Technologies Co.) according to the recommendations of the manufacturer.

Briefly, after initial denaturation at 50 °C for 2 min and 95 °C for 10 min, reactions were run for 40 cycles using the following parameters for all genes studied: 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 45 s. SYBR Green (Applied Biosystems) fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. The primers used for the amplification of cDNAs of interest were synthesized by Integrated DNA Technologies, Inc. The sequences of the forward and reverse primers, respectively, were as follows: 5′-CTT GGA GGT GGC TAC GG-3′ and 5′-CTG GCT GCT CTG GTT CTG-3′ for *Dio1*; 5′-TTC TCC AAC TGC CTC TTC CTG-3′ and 5′-CCC ATC AGC GGT CTT CTC C-3′ for *Dio2*; 5′-TGT TTG ACA ACG GCA GCA TTT-3′ and 5′-CCG AGG CAA CAG TTG GGT A-3′ for the *36B4*, housekeeping gene. We determined relative mRNA levels (2Δ*C*t) by comparing the PCR cycle threshold (*C*t) between groups. Results are expressed relative to the values for the control group, which were considered to be equal to 1.

Determination of TR (TRβ1) and UCP1 content by western blotting analysis

The expression of TRβ1 in liver was examined and UCP1 was detected in BAT. To obtain cell extracts with adequate protein concentrations, we homogenized the tissues in 250 or 500 μl of ice-cold lysis buffer (50 mM HEPES, 1 mM $MgCl_2$, 10 mM EDTA, and 1% Triton X-100, pH 6.4). Inhibitor cocktail (1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml soybean trypsin inhibitor) was added to the lysis buffer at 0.1%. The homogenates were centrifuged at 4 °C and 1120 ***g*** for 5 min. Protein concentrations of the supernatants were determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and samples 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. The supernatants were analyzed by the SDS–PAGE method, using a 10% polyacrylamide gel and 10 μg of total proteins in each gel slot, electroblotted onto a nitrocellulose membrane (Hybond P ECL membrane; Amersham Pharmacia Biotech). The membranes were incubated with 5% nonfat milk in Tween–TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, and 0.1% Tween-20) for 90 min to block nonspecific binding sites. Next, the membranes were washed with TBS and incubated overnight with the following primary antibodies: goat polyclonal anti-TRβ1 diluted 1:1000 (catalog number sc-10822, Santa Cruz Biotechnology) and rabbit policlonal anti-UCP1 diluted 1:500 (catalog number U6382, Sigma–Aldrich). Subsequently, membranes were washed and incubated with secondary antibody (donkey anti-goat IgG–HRP diluted 1:5000 for TRβ1; Santa Cruz Biotechnology and anti-rabbit IgG (WM) Biotin diluted 1:10 000, followed by ExtrAvidin-Peroxidase diluted 1:10 000 for UCP1,

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Table 1 Serum TSH and thyroid hormones of adult rats whose mothers received nicotine during lactation. Values represent mean±S.E.M. of 12 rats/group

	180-day-old offspring	
	C	NIC
TSH (ng/ml)	1.28±0.15	0.82±0.14*
Free T_3 (pg/ml)	2.46±0.14	1.85±0.09*
Free T_4 (ng/dl)	2.51±0.09	2.14±0.08*

TSH, thyrotropin; T_3, triiodothyronine; T_4, thyroxine; C, adult offspring whose mothers were saline-exposed during lactation. NIC, adult offspring whose mothers were nicotine-exposed during lactation. *$P<0.05$.

Sigma–Aldrich) for 1 h at room temperature. After that, immunoreactive proteins were visualized by exposure to X-ray film. Densities of TRβ1 and UCP1 bands were also quantified using Image J 1.34s Software.

TRH and TSH immunohystochemistry

At PN180, NIC and C offspring underwent perfusion (six rats per group, one rat per litter) after 12-h fasting. They were anaesthetized with avertin (0.3 mg/kg i.p.) and intracardial perfusion was performed with saline solution followed by 4% paraformaldehyde and then paraformaldehyde plus 10% sucrose for cryoprotection. Brains and pituitary were sectioned at 20 μm using a cryotome at −20 °C. All coronal sections containing the hypothalamus were collected starting from bregma −1.88 mm, according to Paxinos & Watson (1998). For immunohistochemical procedures, sections were treated with a 0.3% PBS–Triton solution followed by incubation with a blocking solution (5% BSA) and then submitted to immunolabeling using primary antibodies. The brain sections were incubated with anti-TRH produced in rabbits (LifeSpam Biosciences, Seattle, WA, USA; diluted 1:100, catalog number LS-C76393). Pituitaries were incubated with anti-TSH produced in rabbits (Millipore, Darmstadt, Germany; diluted 1:100, catalog number sc-7815). In control procedures, omission of primary antibodies with inclusion of the secondary antibody produced no labeling. Primary antibodies were revealed by incubation with donkey anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes, Eugene, OR, USA) and sections were counterstained with DAPI (from Sigma, diluted 1:5000). The slides were mounted in ProLong Gold antifading reagent (Invitrogen, Molecular Probes).

Image capture was performed by epifluorescence microscopy (Olympus BX-40). For the quantification of hypothalamic sections, we used captured images of four coronal section per animal. We analyzed the paraventricular nucleus (PVN) of the hypothalamus. Images from two fields for each pituitary (four slices per animal) were captured for TSH quantification. We used the segmentation tool from Image-Pro Plus (version 4.5; Media Cybernetic, Inc., Rockville, MD, USA) in quantification of TRH and in TSH analyses. Anti-TRH antibody labels were specific to neuron fibers. As the cut-off point of the immunostaining was selected by the experimenter (who was blind as to group assignment), the segmentation tool procedure was repeated three times, on separate occasions, for each image. The results are expressed as pixel density. For the quantification of TSH, we also counted the number of TSH-positive cells in captured images (four sections counterstained with DAPI per animal).

Pituitary explants: *in vitro* TSH release in response to TRH

The *in vitro* TSH secretion in response to TRH was examined as previously described (Lisboa *et al.* 2008). Pituitaries of NIC and C offspring were quickly dissected out; the anterior pituitary was separated from the posterior pituitary and transected with a longitudinal midline cut. Each anterior hemi-pituitary was immediately transferred to a tube containing 1 ml of minimum essential

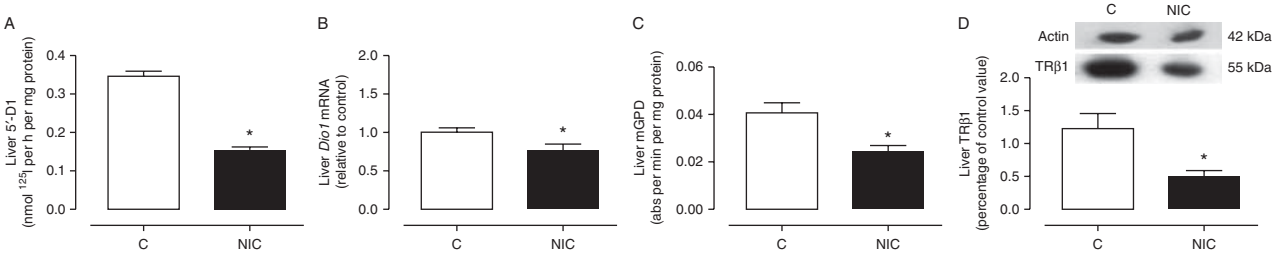

Figure 1
5′-D1 activity (A), *Dio1* mRNA (B), mGPD activity (C), and TRβ1 content (D) in livers of adult offspring whose mothers were exposed to nicotine or saline during lactation. $n=12$ rats/group. Data are presented as mean±S.E.M. *$P<0.05$ versus C.

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medium (MEM), and incubated at 37 °C in an atmosphere of 95% O_2 and 5% CO_2 in a Dubnoff metabolic shaker (50 cycles/min). After a 20 min preincubation period, the medium was removed and hemi-pituitaries were suspended in 1 ml of fresh medium. After 60 min, aliquots were collected for determination of basal TSH release. Next, TRH (Sigma) was added at a final concentration of 50 nM and, after 30 min incubation, aliquots were collected for determination of TRH-stimulated TSH release. The TSH in the medium was measured by RIA. The results are expressed as ng/ml of TSH levels under basal conditions and as the difference between TSH release before and after TRH incubation.

Statistical analyses

The results are expressed as mean ± S.E.M. The GraphPad Prism 5 program (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses and graphics. Data were analyzed by Student's unpaired *t*-test, except for the TSH, which was analyzed by Mann–Whitney *U* test. The significance level was set at $P<0.05$.

Results

As previously reported, adult offspring of dams exposed to nicotine (PN180) showed lower serum TSH, T_3, and T_4 levels (−36, −25, −15%; $P<0.05$ – Table 1).

The offspring of dams in the NIC group presented lower 5′-D1 activity (−53%; $P<0.05$ – Fig. 1A), *Dio1* mRNA (−36%; $P<0.05$ – Fig. 1B), mGPD activity (−41%; $P<0.05$ – Fig. 1C), and TRβ1 content (−62%; $P<0.05$ – Fig. 1D) in liver. The NIC group exhibited higher 5′-D2 activity and expression in BAT (5.2- and 1.2-fold increase; $P<0.05$ – Fig. 2A and B respectively), lower muscle D1 activity and expression (−47 and −25%; $P<0.05$ – Fig. 2C and D), higher heart D2 activity and expression (+35 and +93%; $P<0.05$ – Fig. 2E and F), and higher testis D2 activity and expression (+39 and 47%; $P<0.05$ – Fig. 2G and H).

Postnatal NIC exposure did not change deiodinase activities in hypothalamus, pituitary, and thyroid (Fig. 3). However, UCP1 protein expression in BAT was lower in offspring of the NIC dams (−26%; $P<0.05$ – Fig. 4).

Offspring of dams in the NIC group showed reduced immunoreactivity for TRH compared with offspring of dams in the C group, as determined by the results of both qualitative (Fig. 5B and D) and quantitative analyzes indicated by fiber density (−60%; $P<0.005$ – Fig. 5E). TSH data indicated no differences in quantitative analyses concerning the number of TSH-positive cells between the groups (Fig. 6C). However, TSH cells of offspring of NIC group dams were smaller than those of offspring of C group dams (Fig. 6A and B). This result was confirmed by the quantification of pixel density, which revealed a reduction in TSH immunostaining in the NIC group (−44.9%; $P<0.05$ – Fig. 6D).

The TSH content in the pituitary gland was lower in the offspring of NIC dams (−68%; $P<0.05$ – Fig. 7A). The *in vitro* TSH results are illustrated in Fig. 7B and C.

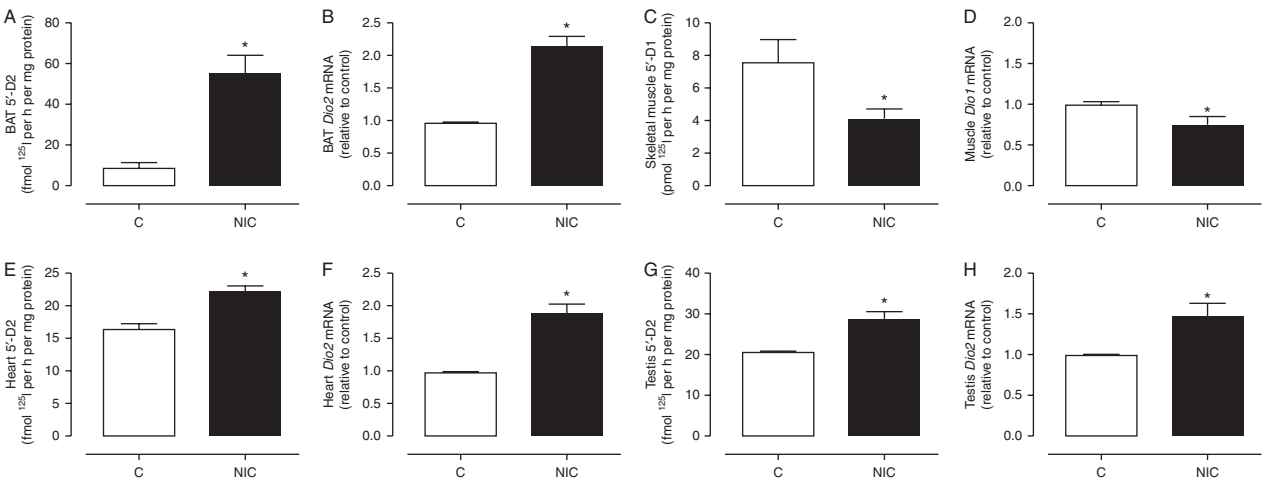

Figure 2
BAT 5′-D2 activity (A), BAT *Dio2* mRNA (B), skeletal muscle 5′-D1 activity (C), skeletal muscle *Dio1* mRNA (D), heart 5′-D2 activity (E), heart *Dio2* mRNA (F), testis 5′-D2 activity (G), and testis *Dio2* mRNA (H) of adult offspring whose mothers were exposed to nicotine or saline during lactation. Data are presented as mean ± S.E.M. *$P<0.05$ versus C, $n=12$ animals/group.

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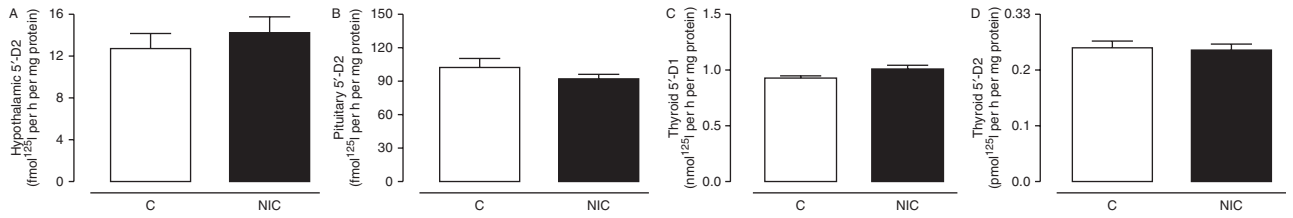

Figure 3
Hypothalamic 5′-D2 (A), pituitary 5′-D2 (B), thyroid 5′-D1 (C), and thyroid 5′-D2 (D) activities of adult offspring whose mothers were exposed to nicotine or saline during lactation. Data are presented as mean ± S.E.M. $n = 12$ animals/group.

Pituitaries from the NIC group presented an increase in basal TSH release (+83%; $P < 0.05$ – Fig. 7B). However, these animals had a lower TSH release in response to TRH compared with animals of the C group (−52%; $P < 0.05$ – Fig. 7C), that is, the TSH secretion was not increased after TRH stimulation, as observed in the control animals.

Discussion

Previously we demonstrated that maternal nicotine exposure during the period of lactation causes primary thyroid hypofunction (lower serum T_4 and T_3 levels with higher TSH level) in the suckling pups and causes overweight, hyperleptinemia, and secondary hypothyroidism (lower TSH, T_4, and T_3 levels) in the offspring in adult life (Oliveira *et al.* 2009). In this study, the lower 5′-D1, mGPD activities, *Dio1* mRNA, and TRβ1 content in the liver at PN180 are in accordance with the hypothyroid status of the adult offspring. In addition, the lower UCP1 in BAT is indicative of a failure in thermogenesis, consistent with hypothyroidism. NIC offspring displayed lower 5′-D1 activity in muscle and higher 5′-D2 activity in BAT, heart, and testis, which are also in accordance with hypothyroidism (Oliveira *et al.* 2009). In all analyzed tissues, deiodinase mRNAs displayed the same profile of enzymatic activity, demonstrating that the hypothyroidism observed in NIC animals, although moderate, is functional, contributing to a hypometabolic profile of programed animals that had central obesity.

In contrast to peripheral TH metabolism, and despite the lower TRH, TSH, and TH levels, adult NIC group animals surprisingly did not present a higher deiodinase activity in the hypothalamus and pituitary. Thus, it is possible that the intracellular TH was preserved in this programing model by higher TH uptake by transporters that include Na^+/taurocholate co-transporting polypeptide (NTCP), monocarboxylate transporter (MCT), and organic anion transporter polypeptides (OATP). Our results indicate that the offspring of NIC dams may develop an adaptive mechanism ensuring an adequate intracellular source of TH in those central tissues, despite lower levels of TRH and TSH, which are possibly compromised by a deficiency in biosynthesis and/or higher TRβ2 expression.

It is well known that leptin upregulates TRH (Légrádi *et al.* 1997) and TSH (Ortiga-Carvalho *et al.* 2002) production *in vivo*. On PN180, NIC offspring had hyperleptinemia and hypothalamic leptin resistance due to lower levels of OB-R, JAK2, p-STAT3 and higher levels of SOCS3 expression (de Oliveira *et al.* 2010), and lower hypothalamic TRH protein content (Younes-Rapozo *et al.* 2013) and lower serum TSH levels (Oliveira *et al.* 2009). Accordingly, in this study, we detected a lower immunoreactivity for TRH and TSH in these animals. As it has already been reported that leptin increases hypothalamic D2 activity in hypothyroid rats (Cabanelas *et al.* 2007), the

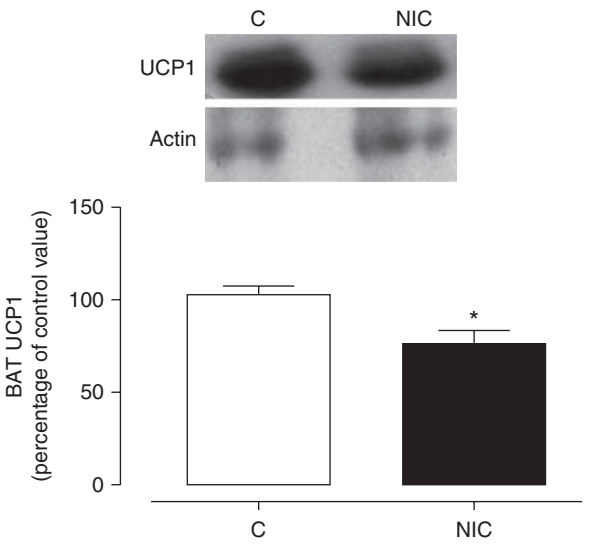

Figure 4
UCP1 in brown adipose tissue of adult offspring whose mothers were exposed to nicotine or saline during lactation. Data are presented as mean ± S.E.M. *$P < 0.05$ versus C, $n = 12$ animals/group.

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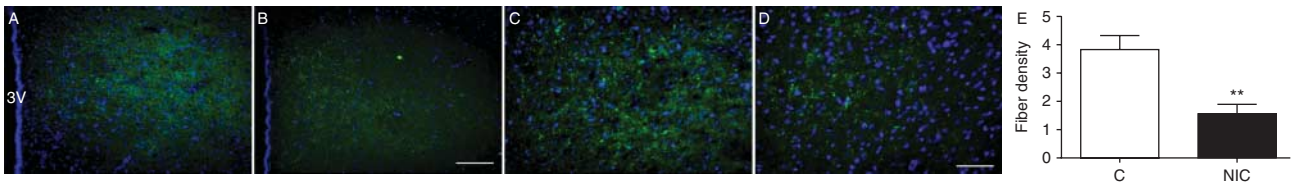

Figure 5
TRH immunohistochemistry in paraventricular nucleus (PVN) of the hypothalamus of adult offspring whose mothers were exposed to nicotine or saline during lactation at PN180. (A, B, C, and D) Imunofluorescence for anti-TRH antibody (revealed with Alexa Fluor 488 – green) and DAPI (blue), (A and C) C offspring, and (B and D) NIC offspring. (C and D) Under higher magnification TRH immunoreactivity is observed to be lower in the NIC group. (E) Quantification of fiber density of TRH immunoreactivity measured by pixel density. Scale bars: 100 μM for A and B and 50 μM for C and D. Data are reported as mean±S.E.M., $^{**}P<0.05$ vs C, $n=6$ animals/group.

unchanged 5′-D2 activity found in the hypothalamus (and also in the pituitary) may be partially due to leptin resistance in NIC offspring. The same fact may explain the lower TSH and TRH tissue content found in these animals. Using the same model, we have previously detected an increase in neuropeptide Y (NPY) in the PVN (Younes-Rapozo *et al*. 2013). As NPY inhibits TRH expression in the PVN (Vella *et al*. 2011, Younes-Rapozo *et al*. 2013), it could help to explain the lower pituitary TSH content in the NIC group. Interestingly, this understimulated pituitary, when incubated *in vitro*, releases more TSH than control pituitaries, indicating that some *in vivo* inhibitory factor is acting on the offspring of NIC dams (e.g., somatostatin). In fact, it has been demonstrated that leptin inhibits somatostatin in hypothalamic cultures (Saleri *et al*. 2004). As the NIC group displays leptin resistance, levels of somatostatin, although not measured, could be higher in our study. Other substances could have the same effect, such as corticosterone, which is increased in offspring of NIC dams (Pinheiro *et al*. 2011).

Although thyroid deiodination is mainly regulated by TSH (Ishii *et al*. 1983), we detected no change in thyroidal 5′-D1 and 5′-D2 activities in the NIC group. This group had lower serum and pituitary TSH that, as expected, is responsible for lower levels of serum TH. Another regulator is possibly contributing for the maintenance of these enzymatic activities, such as leptin, as we demonstrated that leptin increases thyroid D1 activity (Lisboa *et al*. 2003*a*) and as levels of thyroid leptin receptor (OBR) are higher (Santos-Silva *et al*. 2010) in the NIC group, in contrast to its expression in the hypothalamus.

Interestingly, in other models of programing, we obtained evidence that nutritional and hormonal factors during lactation, such as protein and energy restriction (Dutra *et al*. 2003, Lisboa *et al*. 2008), hyperleptinemia (Toste *et al*. 2006*a*), and hypoprolactinemia (Bonomo *et al*. 2008) program changes in deiodinase activity in adult life, caused by either hyperthyroidism or hypothyroidism. In these models, in spite of the thyroid status at adulthood, hyperleptinemia during weaning was observed in all cases and also in neonate NIC pups (Oliveira *et al*. 2009) and could be responsible for programing the T_4 to T_3 conversion at both central and peripheral levels.

There are reports of a stimulatory effect of T_3 on leptin expression (Fain & Bahouth 1998, Groba *et al*. 2013). However, we observed hypothyroidism with hyperleptinemia in offspring of NIC dams. Using the same model, Pinheiro *et al*. (2011) detected a higher leptin content in visceral adipose tissue. Thus, it seems that other factors, besides the direct effect of TH, play a role in the regulation of leptin in this model of programing. The developmental

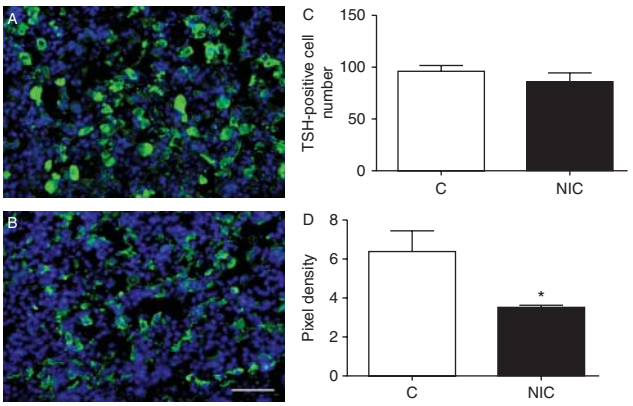

Figure 6
TSH immunohistochemistry in the pituitary of adult offspring whose mothers were exposed to nicotine or saline during lactation. (A and B) Imunofluorescence for anti-TSH antibody (revealed with Alexa Fluor 488 – green) and DAPI (blue), (A) offspring of C group dams; (B) offspring of NIC group dams, (C) quantification of numbers of TSH-positive cells, (D) quantification of density of TSH immunoreactivity indicated by pixel density. The number of TSH-positive cells is not different between the groups; however, TSH immunoreactivity is lower in the NIC group. Scale bars: 50 μm. Data are presented as mean±S.E.M., $^{*}P<0.05$ versus C, $n=6$ animals/group.

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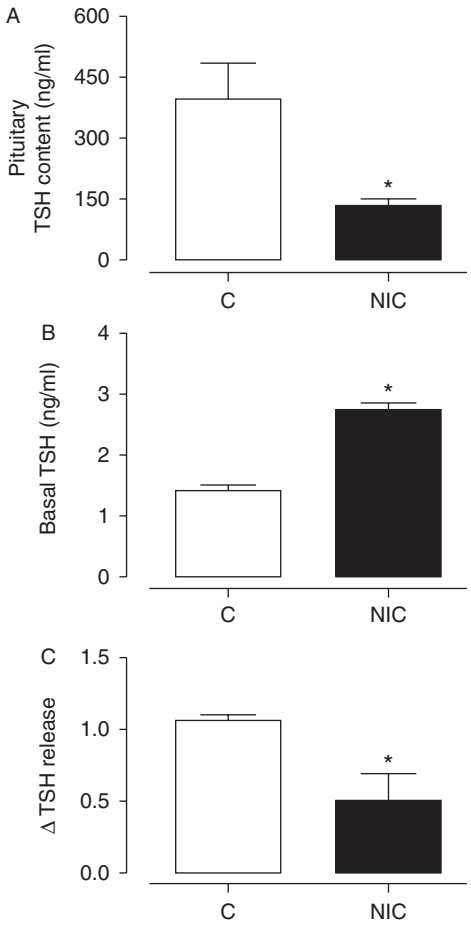

Figure 7
Tissue TSH content (A), medium TSH levels under basal conditions from pituitary gland explants after 60 min (B), Δ=difference between TSH secretion after stimulation with TRH and basal TSH (C) of adult offspring whose mothers were exposed to saline or nicotine during lactation. Data are reported as mean±S.E.M., *$P<0.05$ vs C, $n=6$ pituitaries from six animals per group.

plasticity implies that one imprinting factor, such as nicotine exposure, could affect different systems during development, besides thyroid function.

Briefly, epigenetic mechanisms, such as DNA methylation or histone acetylation/deacetylation induced by pre and/or postnatal environmental factors, may lead to a higher risk of metabolic disturbances during the adult life of the rat progeny (Moura *et al*. 2008). This explanation may help in understanding the mechanisms involved in the long-lasting changes in deiodinase, mGPD, TRβ1, and UCP1 induced by nicotine exposure during the preweaning period. There is a lack of clinical or epidemiological studies regarding hypothyroidism at adulthood programed by neonatal exposure to nicotine. During lactation, nicotine is transferred through the breast milk and can directly act on the newborn's metabolism. Cotinine, the main nicotine metabolite, can be detected in the urine of neonates born to smoking mothers. Smoking mothers have lower iodide contents in their breast milk and urine, and their offspring have lower urinary iodide contents. In smoking mothers, iodine transfer through breast milk is negatively correlated with the concentration of urinary cotinine, the main nicotine metabolite (Laurberg *et al*. 2004). Therefore, it is possible that nicotine reduces iodine transfer through maternal milk. Thus, whether nicotine can make nursing infants exposed to cigarette smoke more susceptible to endocrine and metabolic disorders in adulthood warrants epidemiological investigation.

In summary, the present findings reinforce the concept of the 'developmental origins of health and disease', particularly concerning the programing of thyroid function. During early development, nicotine seems to first affect the thyroid gland, resulting in a hormonal profile in which high TSH levels are a response to the low TH levels. Subsequently, both TRH and TSH levels are reduced generating the thyroid hypofunction in adulthood. Possibly, low levels of TSH are also a result of the action of an *in vivo* inhibitory factor that suppresses its release. Leptin resistance and possibly somatostatin overexpression may play a role in the programming by NIC of thyroid dysfunction. Changes in TH metabolism and function could be the link between exposure to environmental tobacco smoke in early life and later thyroid dysfunctions and obesity.

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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