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# Maternal nicotine exposure during lactation alters hypothalamic neuropeptides expression in the adult rat progeny

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ABSTRACT

Maternal exposure to nicotine during lactation causes hyperleptinemia in the pups and, at adulthood, these animals are overweight and hyperleptinemic, while, in their hypothalamus, the leptin signaling pathway is reduced, evidencing a central leptin resistance. Then, we evaluated the expression of pro-opiomelanocortin (POMC), alpha-melanocyte stimulating hormone (α-MSH), cocaine and amphetamine-regulated transcript (CART), neuropeptide Y (NPY), agouti-related peptide (AgRP) and others in different hypothalamic nuclei in order to better understand the mechanisms underlying the obese phenotype observed in these animals at adulthood. On the 2nd postnatal day (P2), dams were subcutaneously implanted with osmotic minipumps releasing nicotine (NIC-6 mg/kg/day) or saline for 14 days. Offspring were killed in P180 and immunohistochemistry and Western blot analysis were carried out. Significance data had $p < 0.05$. Adult NIC offspring showed more intense NPY staining in the paraventricular nucleus (PVN) (+21%) and increased number of POMC-positive cells in the: arcuate nucleus (+33%), as an increase in fiber density of α-MSH in PVN (+85%). However, the number of CART-positive cells was reduced in the PVN (−25%). CRH staining was more intense in NIC offspring (+136%). Orexins and AgRP were not altered. Thus, maternal nicotine exposure changes hypothalamic neuropeptides in the adult progeny that is partially compatible with leptin resistance.



## 1. Introduction

Maternal smoke during pregnancy is considered a risk factor for low birth weight and neurological abnormalities (Butler and Goldstein, 1973; DiFranza and Lew, 1995; Huang et al., 2006; Navarro et al., 1989; Nunes-Freitas et al., 2011; Thompson et al., 2009). For this reason, many women quit smoking during pregnancy (Giglia et al., 2006; Polańska et al., 2006). However, little is known about postpartum maintenance of smoking cessation and relapse. It was shown that most women who stop smoking during gestation relapse during lactation (Hannöver et al., 2008; McBride and Pirie, 1990; O'Campo et al., 1992). Nicotine is the main addictive compound of tobacco smoke. Nicotine is considered as an 'endocrine disruptor', in other words, can mimic or interfere with the normal action of hormones (Grun and Blumberg, 2006; Tabb and Blumberg, 2006). This fact can be critical during lactation, as nicotine can be transferred through maternal milk (Dahlstrom et al., 1990; Luck and Nau, 1987; Narayanan et al., 2002; Nel and Morgan, 1996).

Changes in nutrition and hormonal status during development, could permanently affect the progeny. This period, including lactation, is a critical window of plasticity due to its large potential to adapt to the environment (de Moura and Passos, 2005; de Moura et al., 2008; Metcalfe and Monaghan, 2001). This phenomenon has been referred to as "developmental plasticity", "programming" or "Barker hypothesis" (Barker, 2003; Gluckman and Hanson, 2007).

Epidemiological studies have demonstrated a trend to higher serum glucose and an increased prevalence of diabetes in children born to smoking women (Huang et al., 2007; Montgomery and

*Abbreviations:* ACTH, adrenocorticotropic hormone; AgRP, agouti-related peptide; ARC, arcuate nucleus; C, control group; CART, cocaine and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; GABA, gamma-aminobutyric acid; JAK2, janus tyrosine kinase 2; LH, lateral nucleus; MCH, melanin-concentrating hormone; MSH, melanocyte-stimulating hormone; nAChR, nicotinic acetylcholine receptor; NIC, nicotine group; NPY, neuropeptide Y; P, postnatal day; POMC, pro-opiomelanocortin; PVN, paraventricullar nucleus; SOCS-3, suppressor of cytokine signaling 3; STAT-3, signal transducer and activator of transcription-3; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; VM, ventromedial nucleus.

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Ekbom, 2002). Other studies, including a meta-analysis with a total of 84,563 children reported in 14 international studies, have showed an increasing risk of overweight and obesity in children, whose mothers smoked during the period of gestation or after delivery (Oken et al., 2008; Somm et al., 2009).

Previously, our group has demonstrated in rats that maternal exposure to nicotine from the 2nd to the 16th day of lactation induced hyperleptinemia, hypothyroidism, higher adrenal catecholamines content in male pups at the end of maternal exposure (Oliveira et al., 2010a). At adulthood, these animals were overweighed and hyperleptinemic, and they also had hypothyroidism, increased medullary adrenal function and serum glucocorticoid level with higher corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (Oliveira et al., 2009, 2010b; Pinheiro et al., 2011) as a consequence of maternal nicotine exposure during the lactation period.

In rats, the hypothalamic circuitry that controls both food intake and energy expenditure is still developing during postnatal life, and disturbances in perinatal nutrition that alter leptin levels may have consequences for the formation and function of these circuitries, affecting food intake and energy expenditure and body weight in adult life (Bouret et al., 2004; Bouret and Simerly, 2004; Coupé et al., 2010). The hypothalamus controls energy homeostasis mainly through neurons in the arcuate nucleus (ARC). This nucleus receives peripheral messages that include satiety and adiposity signals, such as leptin, a molecule that differently regulates two populations of neurons through its long form receptor, Ob-Rb. Anorexigenic neurons releasing pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) are positively regulated by leptin, reducing food intake and increasing catabolic processes; the orexigenic neurons releasing neuropeptide Y (NPY) and agouti-related peptide (AgRP) are negatively regulated by leptin, promoting feeding and energy expenditure inhibition (Sanchez-Lasheras et al., 2010; Valassi et al., 2008). These neurons project their axons to other hypothalamic nuclei such as the ventromedial nucleus (VM), the paraventricular nucleus (PVN) and the lateral hypothalamus (LH), as well as to extrahypothalamic areas. The PVN is particularly important for the releasing of hypothalamic neuro-hormones that control pituitary hormones such as CRH and TRH, both anorexigenic and responsible for increasing energy expenditure. Leptin can act on TRH neurons through NPY, which inhibits TRH expression (Vella et al., 2011), on the other hand, NPY stimulates CRH (Sarkar and Lechan, 2003). Although, both TRH and CRH are anorexigenic, their effects on energy metabolism are different. While TRH through thyroid hormone stimulation increase energy expenditure, CRH acts through glucocorticoid increase on the fat body distribution, increasing central adipogenesis, while decreases subcutaneous fat on the members.

POMC modulates energy homeostasis mainly through one of its cleavage products, α-melanocyte-stimulating hormone (α-MSH), which exerts a tonic inhibitory control on food intake and energy storage though its action on melanocortin receptors (MC3R and MC4R), and AgRP acts as an antagonist of these receptors (Wardlaw, 2011). This system regulates the expression of orexigenic hormones such as melanin-concentrating hormone (MCH) and orexins in the LH [35]. Leptin receptor is also expressed in hypothalamic GABAergic neurons that can regulate the projections of POMC and NPY/AgRP neurons from ARC to PVN. Besides, neurons in PVN are regulated by a complex network of GABAergic inputs, whereby leptin can modulate pre-synaptically POMC activity (Cone and Simerly, 2011; Vong et al., 2011).

In our programming model of nicotine exposure during lactation, we have shown that the total content of ObR in the hypothalamus is reduced in adult offspring. These adult animals also present alteration in the downstream signaling pathway, which are characterized by reductions in the janus tyrosine kinase 2 (JAK2) and in the signal transducer and activator of transcription-3 phosphorylated (p-STAT-3), evidencing a central leptin resistance (Oliveira et al., 2010b).

In view of the metabolic changes observed in the adult progeny in our programming model of postnatal nicotine exposure, including the upregulation in hypothalamic–pituitary-adrenal cortex (HPA) axis activity and the decrease on thyroid function and metabolic rate, we hypothesized that the alterations in the leptin signaling pathway could be responsible for these hormonal changes in this programming model, which could explain in part, why they had normophagia and higher central adiposity. Thus, the goal of this study was to evaluate some aspects of the central mechanisms involved in energy balance regulation such as the expression of orexigenic and anorexigenic neuropeptides in different hypothalamic nuclei, in order to better understand the mechanisms underlying the obese phenotype that is observed in these animals at adulthood.

## 2. Materials and methods

### 2.1. Ethical approval

The use of animals according to our experimental design was approved by the Animal Care and Use Committee of the Instituto de Biologia Roberto Alcantara Gomes da Universidade do Estado do Rio de Janeiro (CEUA/066/2012), which based its analysis on the principles established in the Brazilian Law no. 11.794/2008. Experiments were conducted to minimize the number of animals and the suffering caused by the experimental procedures, following the ethical doctrine of the three "R"s reduction, refinement and replacement (Marques et al., 2009).

### 2.2. Animals

Wistar rats were kept in a temperature-controlled vivarium (25 ± 1 °C) with artificial dark-light cycle (lights on 07:00 a.m., lights off 07:00 p.m.). Pregnant rats were placed in individual cages with free access to water and food. In order to avoid the influence of litter size on the programming, only dams whose litter sizes were at least 10 pups were used. At birth, to maximize lactation performance (Passos et al., 2000), litters were adjusted to 6 male pups per dam.

### 2.3. Experimental model of nicotine programming

After birth, lactating dams were randomly separated into two groups. Dams were lightly anaesthetized with thiopental, an incision on the back was made to allow the subcutaneous insertion of the osmotic minipumps (Alzet, 2ML2, California, USA). Minipump implantation occurred at postnatal day 2 (P2) because according to the manufacturer's recommendation, it must be filled with the solution of interest and immersed in saline for 24 h prior to implantation so as to release substances continuously and homogeneously. The incision was closed and dams were allowed to recover in their home cages.

#### 2.3.1. NIC (nicotine, n = 6)

Pumps were prepared with nicotine free-base diluted in a saline solution (NaCl 0.9%) to deliver an initial dose rate of 6 mg/kg of nicotine per day (during 14 days of lactation), as previously described (Oliveira et al., 2009). Nicotine exposure through subcutaneous osmotic minipump infusion was used to avoid the adverse effects of nicotine peaks. Our protocol produces plasma nicotine levels of approximately 25 ng/mL, similar to those found in typical smokers (Lichtensteiger et al., 1988).

#### 2.3.2. C (controls, n = 6)

Lactating control dams were implanted with osmotic minipumps containing only saline solution, which was also released for 14 days.

After weaning, NIC and C offspring had free access to water and standard laboratory chow. During lactation, body weight was daily monitored and from weaning until P180, body weight and food intake of the offspring were monitored every 4 days. We used one rat from each dam from both groups.

### 2.4. Antibodies

Anti-NPY (rabbit polyclonal antibody, diluted 1:1000) was purchased from Sigma- Aldrich (MO, USA). Anti-alpha-MSH (sheep polyclonal antibody, diluted 1:10,000) was purchased from EMD Millipore Corporation (Billerica, MA, USA). Anti-TRH (rabbit polyclonal antibody, diluted 1:200) was purchased from LifeSpan Biosciences (Seattle, Washington, USA). Anti-CRH (rabbit polyclonal antibody, diluted 1:100) was purchased from Abcam Inc.; Cambridge, MA, USA); The following