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Nicotine exposure during breastfeeding alters the expression of endocannabinoid system biomarkers in female but not in male offspring at adulthood

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Original Article

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Abstract

Early nicotine exposure compromises offspring's phenotype at long-term in both sexes. We hypothesize that offspring exposed to nicotine during breastfeeding show deregulated central and peripheral endocannabinoid system (ECS), compromising several aspects of their metabolism. Lactating rats received nicotine (NIC, 6 mg/Kg/day) or saline from postnatal day (PND) 2 to 16 through implanted osmotic minipumps. Offspring were analyzed at PND180. We evaluated protein expression of N-acylphosphatidylethanolamide-phospholipase D (NAPE-PLD), fatty acid amide hydrolase (FAAH), diacylglycerol lipase (DAGL), monoacylglycerol lipase (MAGL) and cannabinoid receptors (CB1 and/or CB2) in lateral hypothalamus, paraventricular nucleus of the hypothalamus, liver, visceral adipose tissue (VAT), adrenal and thyroid. NIC offspring from both sexes did not show differences in hypothalamic ECS markers. Peripheral ECS markers showed no alterations in NIC males. In contrast, NIC females had lower liver DAGL and CB1, higher VAT DAGL, higher adrenal NAPE-PLD and higher thyroid FAAH. Endocannabinoids biosynthesis was affected by nicotine exposure during breastfeeding only in females; alterations in peripheral tissues suggest lower action in liver and higher action in VAT, adrenal and thyroid. Effects of nicotine exposure during lactation on ECS markers are sex- and tissue-dependent. This characterization helps understanding the phenotype of the adult offspring in this model and may contribute to the development of new pharmacological targets for the treatment of several metabolic diseases that originate during development.

Introduction

Mammals are particularly vulnerable to environmental changes during the lactation period that could induce neuroendocrine adaptations and compromise health at adulthood. The "Development Origins of Health and Diseases" (DOHaD) hypothesis aims to explain the impact of environmental changes during early life stages.[1] Chemicals and drugs exposures are important factors that are capable of programming the progeny to a deregulated metabolism in later life.[2] As an example, nicotine, the major addictive compound present in cigarette smoke, has already been described as a chemical disruptor that changes milk supply and composition,[3,4] affecting the baby's health at short and long term.[5,6] Nicotine is an important emerging pollutant that can be found on surfaces,[7] water,[8] soil, air[9] and even in pregnant woman's hair.[10] We have previously demonstrated that maternal nicotine exposure during lactation compromises rat offspring's phenotype at long-term in a sex-dependent manner: male offspring showed, at adulthood, obesity, normophagia, despite hypothalamic leptin resistance, hyperleptinemia, hypercorticosteronemia and hypothyroidism, while female offspring had normal body mass, despite hyperphagia, unchanged leptinemia and corticosteronemia, but hyperthyroidism.[11-13]

Several different mechanisms have been associated with endocrine dysfunctions, some of which have been linked to obesity onset. Studies have already described an interaction of a disturbed endocannabinoid system (ECS) with endocrine system changes and obesity.[14-16] The ECS acts in many physiological processes, both in the central nervous system (CNS)[17] and in peripheral tissues,[18] via signaling pathways that depend on enzymatic machinery. The endogenous cannabinoids *N*-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are derived from membrane phospholipids and are synthesized by the enzymes *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively.[19] The endocannabinoids act via two G protein-coupled receptors: the cannabinoid receptor type-1 (CB1) and the cannabinoid receptor type-2 (CB2).[20] Once utilized by the cells,

AEA is degraded by the fatty acid amide hydrolase (FAAH) and the 2-AG is degraded by the monoacylglycerol lipase (MAGL).[21] In the CNS, endocannabinoids activity was shown to affect levels of anxiety and depression, neurogenesis, reward, cognition, learning, memory and food intake.[22,23] In the lateral hypothalamus (LH), ECS activation increases food intake[24] and reduces energy expenditure.[25] In the paraventricular nucleus (PVN), besides modulating energy expenditure,[26] endocannabinoids also seem to be involved in neuroendocrine processes, such as the inhibitory effect on the negative feedback of glucocorticoids on corticotropin-releasing hormone (CRH) secretion.[27] In the liver, endocannabinoids increase lipid accumulation and reactive oxygen species.[28] In the white adipose tissue, they are involved in increasing production and storage of triglycerides, reduction of adiponectin synthesis, and mitochondrial biogenesis.[29] In the adrenal gland, ECS influences adrenocortical steroidogenesis[30] and suppresses adrenalin release from adrenal medullary cells,[31] while, in the thyroid gland, it inhibits thyroid hormone release.[32]

Some studies have shown that the ECS modulates the addictive and rewarding properties of nicotine.[33-35] However, to our knowledge, the literature does not report data regarding the effects of nicotine exposure during the lactation period on the ECS of experimental models of metabolic programming. Due to the fact that the ECS modulates energy homeostasis and many endocrine functions, we hypothesize that the dysfunctions previously characterized in the offspring of our experimental model of metabolic programming by maternal nicotine exposure during breastfeeding is associated with a disrupted ECS in the CNS and in of peripheral tissues, such as liver, adipose tissue, adrenal and thyroid glands.

Material and Methods

Ethics approval

The Ethical Committee for Use of Laboratory Animals of the Biology Institute, Rio de Janeiro State University (CEUA/033/2017) has approved all experimental procedures. All animals were housed under controlled conditions in a 12-h light-dark cycle (lights on from 7 a.m. to 7 p.m.) and at a temperature of 21 ± 2°C throughout the experiment. Water and standard rodent chow diet (Nuvilab®, São Paulo, Brazil) were offered *ad libitum*. We performed the experiments in accordance to the American Physiological Society's guiding principles.

Animals and nicotine exposure

Female and male *Wistar* rats were mated at 3 months old. After detection of pregnancy, pregnant rats were housed in individual cages. At birth, all litters were normalized to six pups per litter. At postnatal day (PND) 2, 14 lactating rat dams were randomly assigned to one of the following groups:

I) Nicotine (NIC, $n = 7$): dams were anesthetized with thiopental, a 3 × 6 cm area on the back was shaved and an incision was made to insert an osmotic minipump (OMP, Alzet, 2ML2, Los Angeles, CA, USA) subcutaneously. OMPs were prepared with nicotine free-base (Sigma, St Louis, MO, USA) diluted in NaCl 0.9% to release a dose of 6 mg/kg of nicotine/day from the PND2 to PND16, as previously described[37];

II) Control ($n = 7$): dams were implanted with OMPs containing NaCl 0.9%.

The nicotine exposure via s.c. OMP avoids the adverse effects of nicotine peaks. In this rat model, the regimen of maternal nicotine exposure results in a total exposure of 84 mg/kg in 14 days per dam, which approximates cotinine levels of heavy human smokers.[38] The period of 14 days of exposure during lactation corresponds to approximately 3–4 months of maternal smoking during breastfeeding in humans. Offspring were exposed to nicotine exclusively via milk and, at weaning, the pup's blood cotinine was 20 ng/mL.[39]

Offspring, at PND180, from both sexes, were weighed and anesthetized with thiopental (i.p. 150 mg/kg of body mass) and euthanized by cardiac puncture. Brain, liver, VAT, adrenal and thyroid glands were collected, immediately frozen in liquid nitrogen and stored at −80°C for analyses.

Punch technique

Coronal brain sections were cut using a cryostat (Hyrax C25, Zeiss, Germany) and punches of the lateral hypothalamus (LH, bregma 2.04 to 3.60 mm) and the paraventricular nucleus of hypothalamus (PVN, bregma 0.6 to −2.1 mm) were extracted according to Paxinos and Watson stereotaxic coordinate atlas.[40] The cut thickness was approximately 1500 µm for both nuclei (variations in this parameter occurred as a function of sex, age, and size of the animals).

Western blotting

Frozen tissues were macerated in a specific extract buffer containing a protease inhibitor cocktail (S8830, SIGMAFAST™, Sigma-Aldrich, St Louis, MO, USA). The LH and PVN punches were sonicated twice in an ultrasonic processor for 10 s (15 s interval, 40% amplitude). Liver, VAT, adrenal and thyroid tissues were centrifuged at 17,004 × *g* for 15 min, 12,851 × *g* for 30 min, 10,621 × *g* for 5 min and 15,294 × *g* for 20 min at 4°C (Eppendorf 5417R, Hampton, USA), respectively. Total protein content was determined using a BCA™ Protein Assay Kit (Thermo Scientific®, Rockford, IL, USA). All samples were treated with Laemmli sample buffer (w/v: glycerol 30%; β-mercaptoethanol 20%; sodium dodecyl sulfate (SDS) 8%, 0.25 M Tris at pH 6.8 and bromophenol blue). Total protein extracts (15~20 µg) were separated by 10% SDS-PAGE at 200 V for approximately 50 min. The proteins were then transferred from the gel to a polyvinylidene difluoride (PVDF) membranes using the Trans-Blot® turbo system (Bio-Rad® Laboratories, Hercules, CA, USA) and blocked with 5% BSA in Tween-Tris-buffered saline (TTBS; Tris-HCl, 1 mol/L; NaCl, 5 mol/L; and Tween 20, 0.05%, v/v) for 90 min under continuous shaking. Membranes were incubated overnight with the primary antibodies described in Table 1. PVDF filters were washed 3 times with Tween–TBS (0.1%), followed by 1 h incubation with appropriate biotin-conjugated secondary antibody (Table 1). Then, membranes were incubated with streptavidin-conjugated HRP (RPN1231V; Sigma-Aldrich, St Louis, MO, USA). Immunoreactive proteins were visualized with an ECL kit using an Image Quant LAS (Bio-Rad® Laboratories, Hercules, CA, USA). Bands were quantified by densitometry using Image J 1.4 software (Wayne Rasband, National Institutes of Health, Bethesda, MA, USA). Protein contents of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the LH and PVN, as well as cyclophilin B in the liver, VAT, adrenal and thyroid, were used as loading controls.

Statistical analyses

Data are expressed as mean ± standard error of the mean and were analyzed with the statistical program GraphPad Prism 6.0 (San Diego, CA, USA). Each variable was analyzed using two-way

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Table 1. Antibodies used in western blotting

Primary Antibodies			Secondary antibodies		
Antibody	Catalogue number/ Distributed by	Dilution	Distributed by	Dilution	Specificity
CB1	ab23703 / Abcam, UK	1:200	Sigma-Aldrich, USA	1:7000	Anti-rabbit
CB2	ab3561 / Abcam, UK	1:1000	Sigma-Aldrich, USA	1:10000	Anti-rabbit
Cyclophilin B	PA1-027A / Thermo Fisher Scientific, USA	1:1000	Sigma-Aldrich, USA	1:10000	Anti-rabbit
DAGL-α	ab81984 / Abcam, UK	1:500	Sigma-Aldrich, USA	1:20000	Anti-goat
FAAH	ab54615 / Abcam, UK	1:400	Sigma-Aldrich, USA	1:10000	Anti-mouse
GAPDH	14C10 #2118 / Cell Signaling Technology, USA	1:1000	Sigma-Aldrich, USA	1:10000	Anti-rabbit
MAGL	sc-134789 / Santa Cruz Biotechnology, USA	1:400	Sigma-Aldrich, USA	1:7000	Anti-rabbit
NAPE-PLD	ab95397 / Abcam, UK	1:1000	Sigma-Aldrich, USA	1:10000	Anti-rabbit

CB1: cannabinoid type-1 receptor; CB2: cannabinoid type-2 receptor; DAGL-α: diacylglycerol lipase alpha; FAAH: fatty acid amide hydrolase; GAPDH: glyceraldehyde-3-phosphate 5 dehydrogenase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

Table 2. ANOVA results regarding nicotine exposure during lactation on endocannabinoid system of the adult offspring

	ANOVA – Group × Sex interactions					
	NAPE-PLD	FAAH	DAGL	MAGL	CB1	CB2
Lateral hypothalamus	F (1, 23) = 0.1, $p > 0.10$	F (1, 24) = 0.7, $p > 0.10$	F (1, 24) = 2.5, $p > 0.10$	F (1, 24) = 0.3, $p > 0.10$	F (1, 24) < 0.1, $p > 0.10$	
Paraventricular nucleus	F (1, 22) = 1.4, $p > 0.10$	F (1, 23) = 0.5, $p > 0.10$	F (1, 23) = 1.0, $p > 0.10$	F (1, 22) < 0.1, $p > 0.10$	F (1, 22) < 0.1, $p > 0.10$	
Liver	F (1, 19) = 0.3, $p > 0.10$	F (1, 23) < 0.1, $p > 0.10$	F (1, 23) = 2.1, $p > 0.10$	F (1, 23) = 0.2, $p > 0.10$	F (1, 22) = 1.0, $p > 0.10$	F (1, 21) = 0.4, $p > 0.10$
Visceral adipose tissue	F (1, 21) < 0.1, $p > 0.10$	F (1, 20) = 0.8, $p > 0.10$	F (1, 19) = 7.7, $p = 0.012$	F (1, 23) < 0.1, $p > 0.10$	F (1, 23) = 0.5, $p > 0.10$	F (1, 20) = 1.4, $p > 0.10$
Adrenal gland	F (1, 21) = 3.3, $p = 0.083$	F (1, 24) < 0.1, $p > 0.10$	F (1, 23) < 0.1, $p > 0.10$	F (1, 23) = 1.1, $p > 0.10$	F (1, 23) = 0.2, $p > 0.10$	
Thyroid gland	F (1, 22) = 0.1, $p > 0.10$	F (1, 21) = 9.7, $p = 0.005$	F (1, 24) = 1.2, $p > 0.10$	F (1, 22) = 0.3, $p > 0.10$	F (1, 23) < 0.1, $p > 0.10$	

CB1: cannabinoid type-1 receptor; CB2: cannabinoid type-2 receptor; DAGL-α: diacylglycerol lipase alpha; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

ANOVA with group and sex as between-subject factors (Table 2). Considering that, for the Western blotting analyses, males and females were assessed in different gels, the results in the graphs were analyzed separated by sex using Student's t-tests (Control *vs.* NIC) for each protein. Differences were considered significant when $p < 0.05$. Effect size data is provided as Eta-squared (η^2: small > 0.1, medium > 0.3, large > 0.5).

Results

NIC males showed a significant increase in body mass (595 ± 15 g) when compared to control males (520 ± 10 g), while NIC females showed no difference (NIC: 300 ± 8 *vs.* CON: 295 ± 10 g) when compared to control females. This finding agrees with findings of our previous studies.[11-13]

ECS markers in the lateral hypothalamus (LH) and paraventricular nucleus (PVN)

As shown in Fig. 1, the protein levels of the ECS markers in the LH were not affected by nicotine exposure in both male (Fig. 1*a* and 1*b*) and female offspring (Fig. 1*c* and 1*d*).

We did not observe significant differences between groups in the parameters of the ECS in the PVN in both male (Fig. 2*a* and 2*b*) and female offspring (Fig. 2*c* and 2*d*). In both nuclei, the ANOVAs failed to identify interactions between early nicotine exposure and sex (Table 2).

ECS markers in the liver

No significant differences were observed between groups in the ECS biomarkers of the male offspring (Fig. 3*a* and 3*b*). In contrast, NIC females had significantly lower DAGL (−44%, $p = 0.012$, $\eta^2 = 0.450$) and CB1 protein levels (−40%, $p = 0.028$, $\eta^2 = 0.396$) in the liver (Fig. 3*c* and 3*d*) in comparison with controls. No interactions between early nicotine exposure and sex were observed (Table 2).

ECS markers in the visceral adipose tissue (VAT)

NIC males did not show any alteration in ECS markers (Fig. 4*a* and 4*b*) in the VAT when compared to control males. Conversely, NIC females showed a significant increase in DAGL protein level (2.6-fold, $p = 0.002$, $\eta^2 = 0.611$) (Fig. 4*c* and 4*d*) in this tissue, in line with the observation of a significant interaction between Sex and Group for this marker (Table 2).

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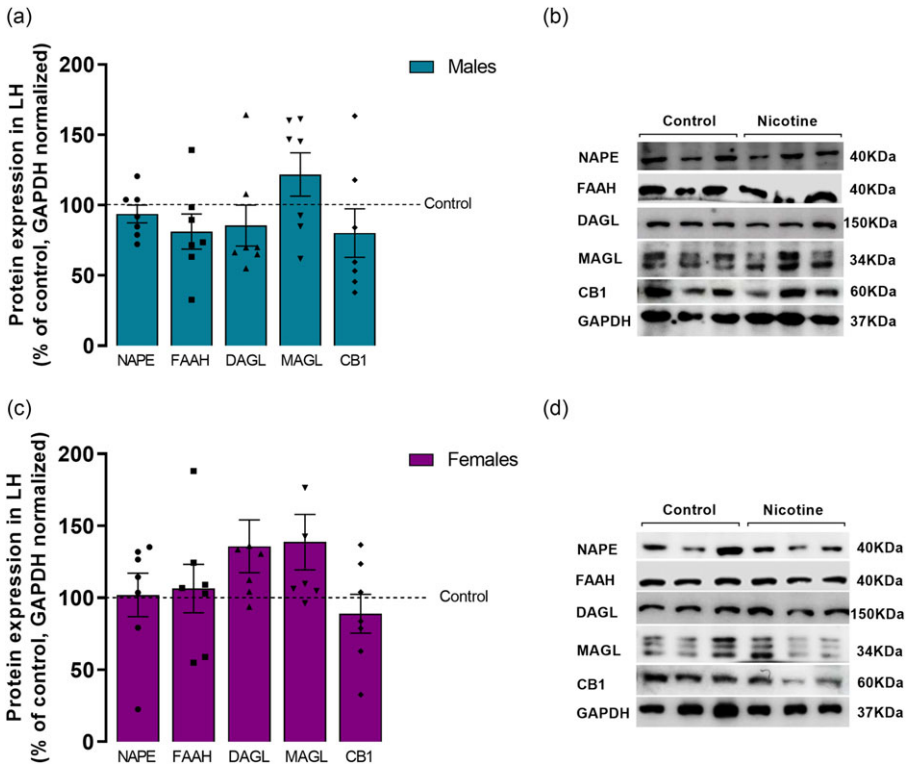

Fig. 1. Effects of nicotine exposure during breastfeeding on the endocannabinoid system in the lateral hypothalamus (LH) of male (*a*) and female (*c*) offspring at PND180. Representative western blot bands for each protein are shown (*b*; *d*). GAPDH was used as housekeeping gene. Data are expressed as mean ± S.E.M, $n = 7$ animals from different litters/group. CB1: cannabinoid type-1 receptor; DAGL: diacylglycerol lipase; FAAH: fatty acid amide hydrolase; GAPDH: glyceraldehyde-3-phosphate 5 dehydrogenase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

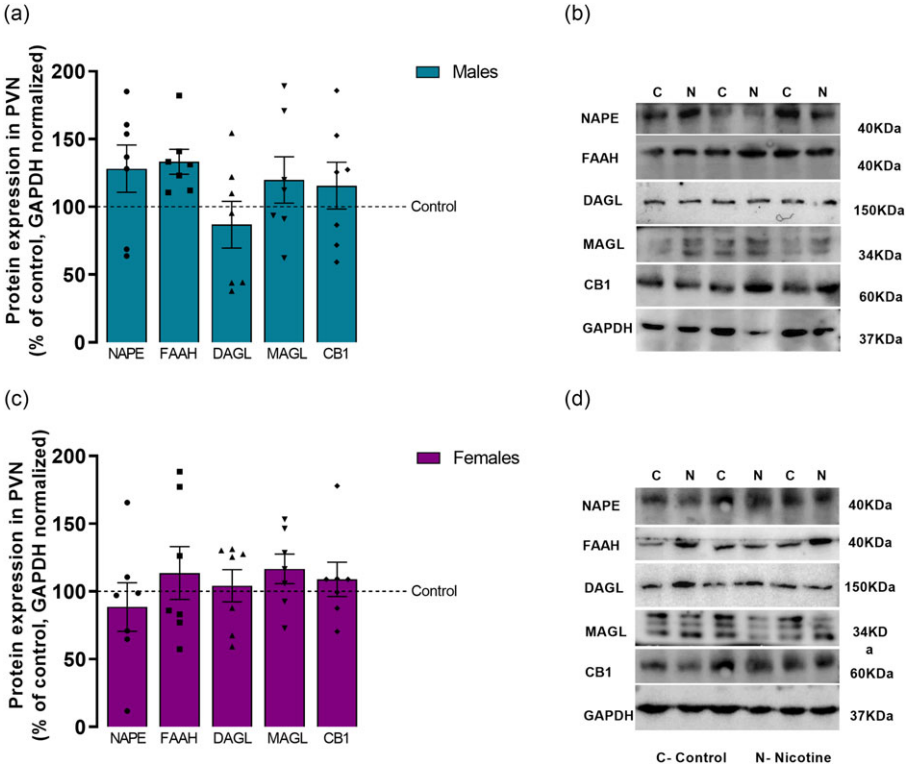

Fig. 2. Effects of nicotine exposure during breastfeeding on the endocannabinoid system in the paraventricular nucleus (PVN) of male (*a*) and female (*c*) offspring at PND180. Representative western blot bands for each protein are shown (*b*; *d*). GAPDH was used as housekeeping gene. Data are expressed as mean ± S.E.M, $n = 7$ animals from different litters/group. CB1: cannabinoid type-1 receptor; DAGL: diacylglycerol lipase; FAAH: fatty acid amide hydrolase; GAPDH: glyceraldehyde-3-phosphate 5 dehydrogenase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

ECS markers in the adrenal glands

Nicotine exposure during breastfeeding did not program the ECS markers in adrenal glands of male offspring (Fig 5*a* and 5*b*). Conversely, NIC females had a significant increase in NAPE-PLD protein level (2.5-fold, $p = 0.026$, $\eta^2 = 0.438$) in the adrenal glands (Fig. 5*c* and 5*d*), a result compatible with the significant Sex × Group interaction for this marker (Table 2).

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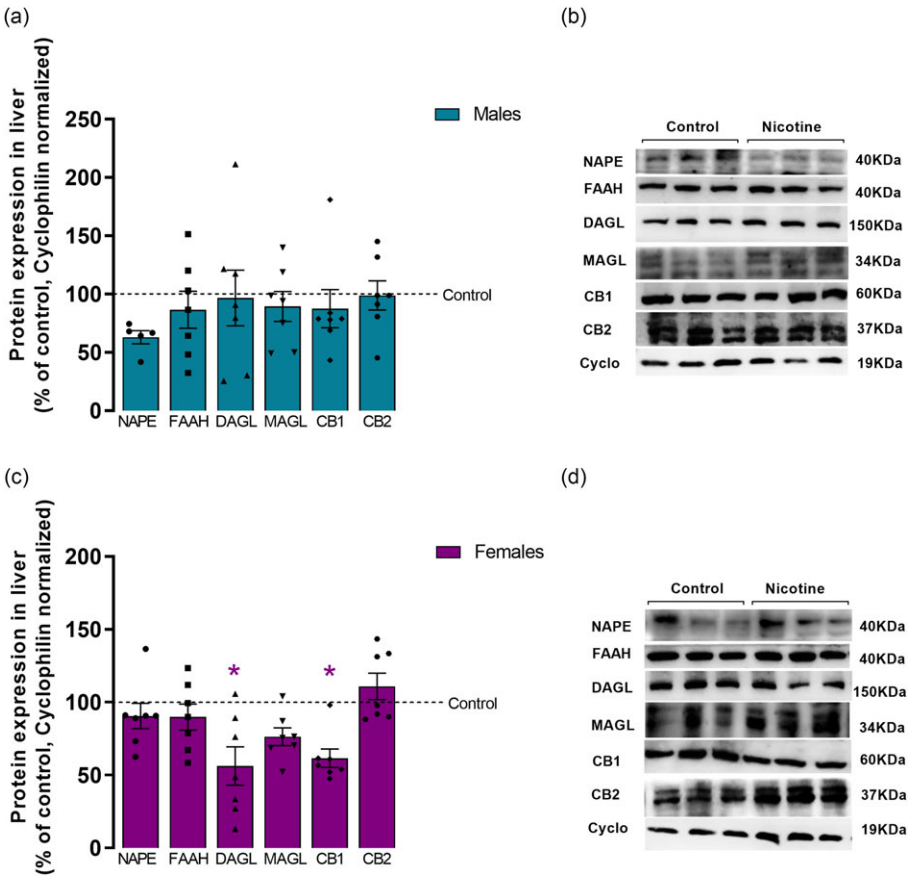

Fig. 3. Effects of nicotine exposure during breast-feeding on the endocannabinoid system in the liver of male (*a*) and female (*c*) offspring at PND180. Representative western blot bands for each protein are shown (*b*; *d*). Cyclophilin was used as housekeeping gene. Data are expressed as mean ± S.E.M, $n = 7$ animals from different litters/group, except for NAPE-PLD from males, $n = 5$ animals from different litters/group. Differences are represented by *, considering $p < 0.05$, (Student's *t*-test); $^{*}p = 0.012$ (DAGL) and $^{*}p = 0.028$ (CB1). CB1: cannabinoid type-1 receptor; CB2: cannabinoid type-2 receptor; Cyclo: Cyclophilin; DAGL: diacylglycerol lipase; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

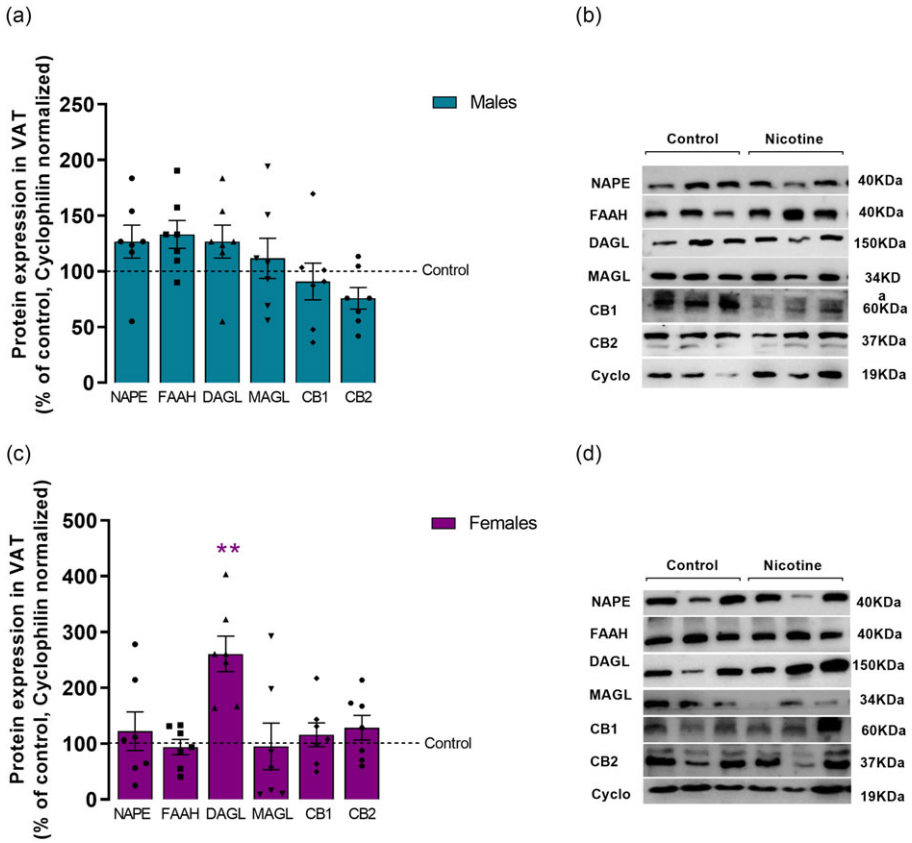

Fig. 4. Effects of nicotine exposure during breast-feeding on the endocannabinoid system in the visceral adipose tissue (VAT) of male (*a*) and female (*c*) offspring at PND180-day-old. Representative western blot bands for each protein are shown (*b*; *d*). Cyclophilin was used as housekeeping gene. Data are expressed as mean ± S.E.M, $n = 7$ animals from different litters/group. Differences are represented by **, considering $p < 0.01$ (Student's *t*-test); $p = 0.002$. CB1: cannabinoid type-1 receptor; CB2: cannabinoid type-2 receptor; Cyclo: Cyclophilin; DAGL: diacylglycerol lipase; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

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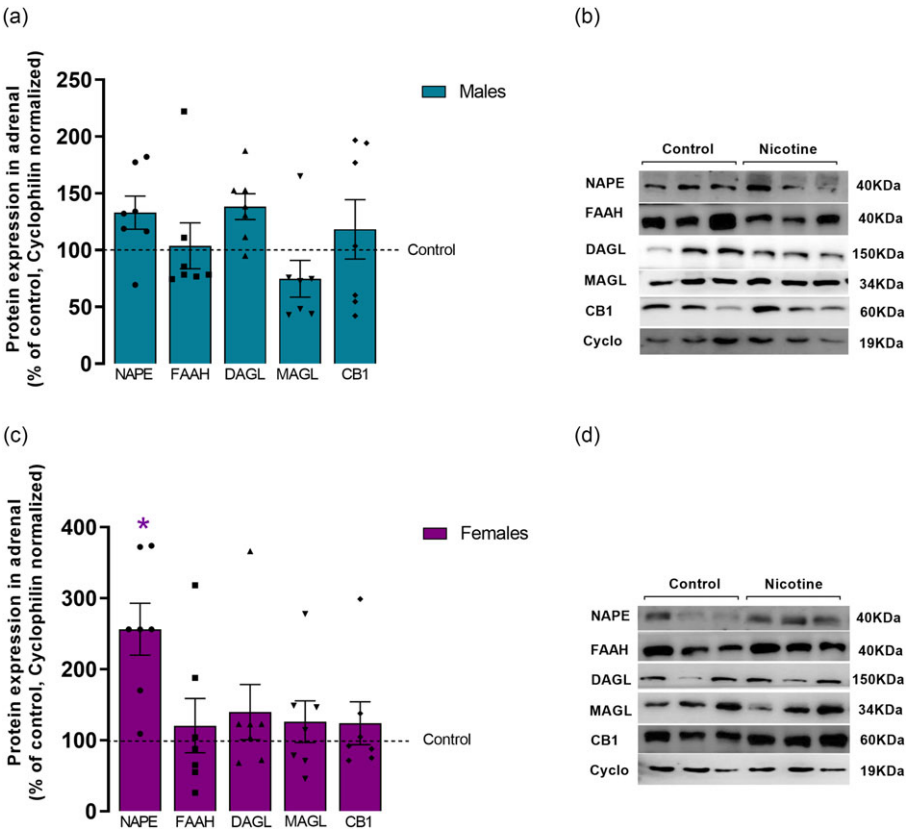

Fig. 5. Effects of nicotine exposure during breastfeeding on the endocannabinoid system in the adrenal glands of male (*a*) and female (*c*) offspring at PND180. Representative western blot bands for each protein are shown (*b*; *d*). Cyclophilin was used as housekeeping gene. Data are expressed as mean ± S.E.M, $n = 7$ animals from different litters/group. Statistical differences are represented by *, considering $p < 0.05$ (Student's *t*-test); $p = 0.026$. CB1: cannabinoid type-1 receptor; Cyclo: Cyclophilin; DAGL: diacylglycerol lipase; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

ECS markers in the thyroid gland

NIC males showed no changes in ECS markers when compared to control males (Fig 6*a* and 6*b*). Only NIC females showed an increased FAAH protein level (2.3-fold, $p = 0.006$, $\eta^2 = 0.586$) in the thyroid gland (Fig. 6*c* and 6*d*), corroborating the Sex × Group interaction that was observed for this marker (Table 2).

Discussion

In this study, for the first time, we characterized the ECS in different tissues in a programming model of nicotine exposure during breastfeeding. The ECS has a relevant role in the modulation of energy expenditure and in many physiological processes. Our main findings demonstrate that the major ECS markers were not affected in the LH and PNV nuclei of both male and female NIC offspring. Conversely, ECS markers of NIC females seem to be more affected in the peripheral tissues, since we characterized significantly lower protein expression of the biomarkers of ECS synthesis and action in the liver, higher DAGL expression (marker for 2-AG production) in the VAT, higher NAPE-PLD expression (marker for AEA synthesis marker) in the adrenal gland and higher FAAH expression (a marker of AEA degradation) in the thyroid gland.

Endocannabinoids are directly related to obesity onset. Several studies have demonstrated that the permanent overstimulation of the ECS, mainly in the CNS, contributes to the obese status.[16,41,42] In our experimental model, NIC male offspring were imprinted to an obese phenotype at adulthood. It is therefore conceivable that the nicotine exposure during breastfeeding has modulated the offspring ECS. Indeed, the ECS was impaired in the CNS, specifically in dorsolateral striatum region, of adult Wistar rats that were exposed to nicotine, even after a long period of nicotine withdrawal.[43] Although this study did not evaluate the metabolic programming model or the same tissues that were assessed here, we suggest that an early nicotine exposure, during a phase of great CNS vulnerability, is capable of affecting the ECS at adulthood by imprinting a disruption of its activity. Interestingly, the imprinting process seems to be sex-dependent since only females showed marked effects.

Regarding the specific regions in the CNS in which the ECS plays important roles, such as the regulation of food intake, control of energy expenditure and neuroendocrine processes,[24,26,27] both the LH and the PVN must be highlighted. In the current study, we did not observe changes in ECS markers in the LH of both male and female offspring. This result is compatible with NIC males normophagia but not with females hyperphagia, as shown in our previous results,[44] which indicates that the endocannabinoid pathway did not contribute to this female phenotype. Other pathways, involving dopaminergic tonus or central leptin resistance, may explain this hyperphagia in NIC females.[13] In the PVN, we did not observe changes in ECS markers in the offspring of either sex. ECS function could be associated with the regulation of glucocorticoid negative feedback in the PVN since NIC male offspring exhibit higher corticotropin-releasing hormone (CRH), higher adrenocorticotropic hormone and hypercorticosteronemia, as previously demonstrated by our group.[44] In the PVN, the feedback mechanism is mediated by a non-genomic corticosterone binding to glucocorticoid receptor and involves the endocannabinoid synthesis.[45] In addition, pharmacological inhibition of CB1 receptors results in increased corticosterone concentrations in male and female Wistar rats.[46] Similarly, the endocannabinoid system also acts in the inhibition of thyrotropin-releasing hormone (TRH)

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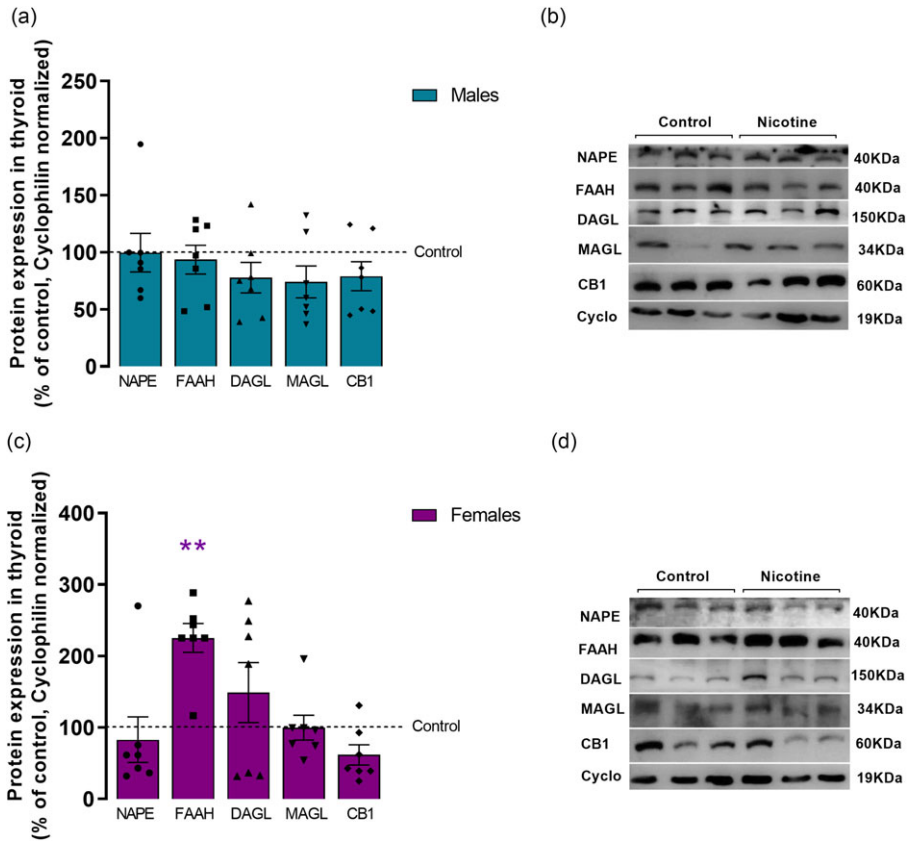

Fig. 6. Effects of nicotine exposure during breast-feeding on the endocannabinoid system in thyroid glands of male (*a*) and female (*c*) offspring at PND180. Representative western blot bands for each protein are shown (*b*; *d*). Cyclophilin was used as housekeeping gene. Data are expressed as mean ± S.E.M, $n = 7$ animals from different litters/group. Statistical differences are represented by **, considering $p < 0.01$ (Student's *t*-test); $p = 0.006$. CB1: cannabinoid type-1 receptor; Cyclo: Cyclophilin; DAGL: diacylglycerol lipase; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acylphosphatidylethanolamine-phospholipase D.

release, as indicated by an experiment that blocked CB1 receptors with an antagonist and observed, as a result, an increased TRH release in mouse median eminence explants.[47]

The deregulation of the ECS does not occur only at a central level, but also in the periphery. Obese people show both central and peripheral ECS increases.[41] Moreover, a deregulated ECS can be involved in other diseases. In the liver, for example, an upregulation of CB1 is related with hepatic fibrosis, steatosis, oxidative stress and lipid accumulation.[48-50] Our group has already demonstrated that NIC males have increased lipid accumulation, oxidative stress and steatosis.[51] However, these dysfunctions in our male experimental model apparently are independent of the ECS function, since ECS tonus was unaltered. In contrast, NIC females had a reduction of DAGL and CB1 receptor in the liver, which may help to protect this tissue from the harmful effects of ECS overstimulation, since NIC females have unchanged hepatic cytoarchitecture, as we have previously shown.[52] It is possible that a compensatory effect is improving liver function by a mechanism remains to be determined.

The ECS also acts in the white adipose tissue stimulating lipogenesis and adipogenesis.[53] In the VAT, NIC males did not show changes in ECS markers, while females had higher DAGL. In most cases, the deregulation of ECS is characterized by their overactivation.[54] The increase in the biosynthetic enzyme DAGL in the female offspring VAT suggests an increased 2-AG synthesis, which could be related with the higher adipocyte area as well as with the increased protein content of acetyl-CoA carboxylase and transcription factor CCAAT/enhancer-binding protein (C/EBP) beta that were previously observed in these animals.[55] It is also known that increased 2-AG biosynthesis is involved in pro-inflammatory and prostaglandin responses, mainly because it serves as substrate for cyclooxygenases.[56,57]

Concerning the ECS in the adrenal gland, NIC males did not show any changes, while NIC females had higher NAPE-PLD, which could indicate higher AEA synthesis. However, it is important to highlight that bioactive N-acylethanolamines, including AEA, can be formed from N-acylated plasmalogen through a NAPE-PLD-independent pathway.[58] Our research group has already described the effects of nicotine exposure during lactation on the adrenal function of the offspring. We observed that males had higher adrenal catecholamine content, but lower *in vitro* catecholamine release,[44] while females had normal adrenal function.[55] Endocannabinoids have an inhibitory effect on adrenaline secretion, as demonstrated by the administration of cannabinoids in isolated rabbit adrenal glands.[31] In the adrenocortical cell line NCI-H295R, it was demonstrated that AEA is capable of inhibiting steroidogenesis.[30] Furthermore, the administration of CB1 antagonist AM251 was shown to increase corticosterone levels in the adrenal cortex.[59] Contrasting with the unaltered expression of adrenal CB1 in our model, Surkin and Gallino[60] suggest that, in the adrenal cortex, AEA has a potent effect via the vanilloid transient receptor potential cation channel subfamily V member 1 (TRPV1 receptors), which was not evaluated here. It is important to highlight that endocannabinoid stress modulation could be bidirectional: stress could alter the ECS activity as well as the ECS activity could induce alterations in HPA axis function.

The ECS is also present in the thyroid gland.[32] Studies suggest that endocannabinoids have a negative regulation on TSH, T3 and T4 secretions.[32,61,62] In addition, FAAH knockout mice had significant TSH, T_3 and T_4 reductions.[63] In a previous work, we

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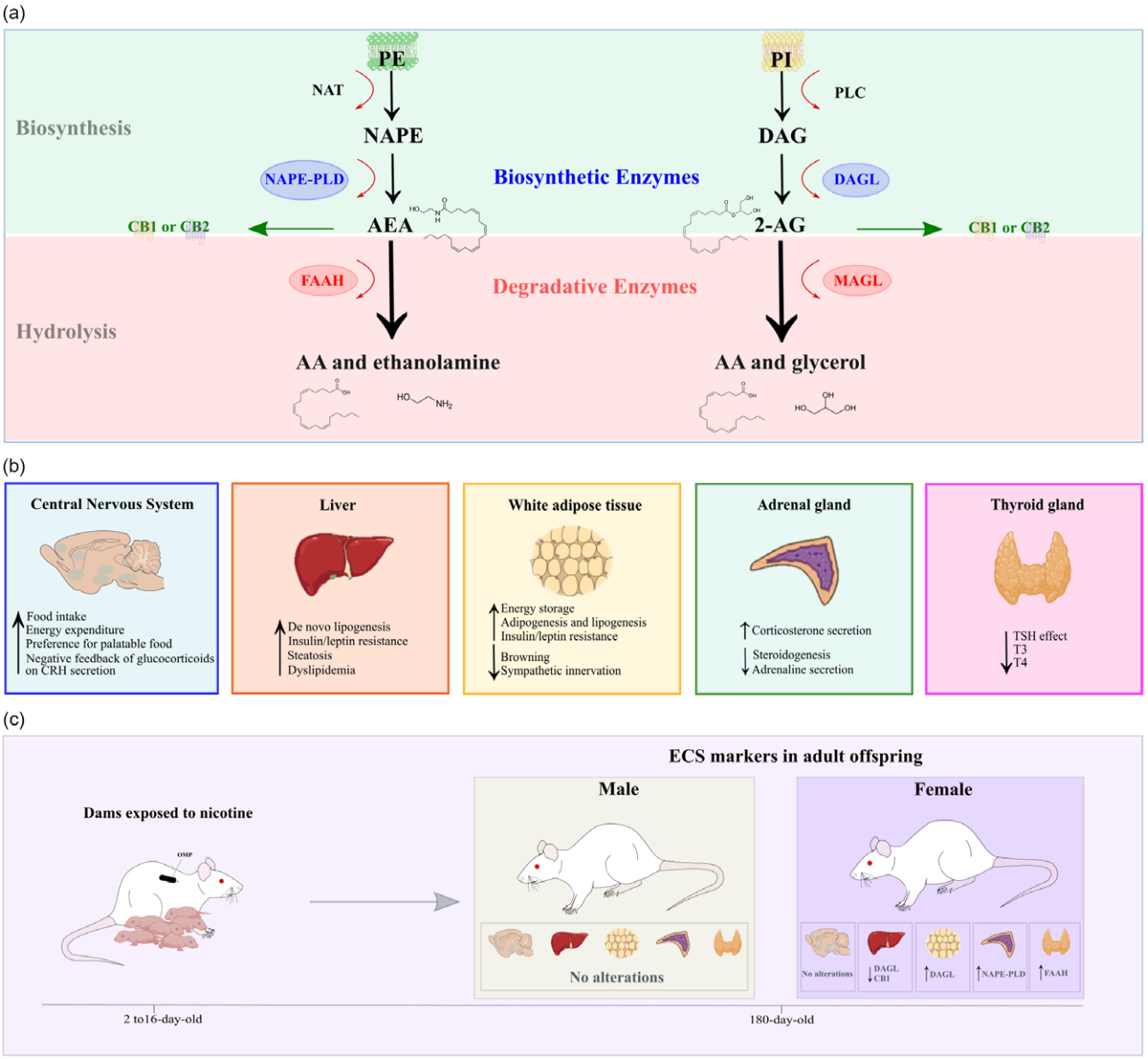

Fig. 7. The first panel (*a*) depicts the main proteins involved in endocannabinoid synthesis and degradation pathways. Phosphatidylethanolamine (PE) is converted to N-acyl-phosphatidylethanolamine (NAPE), catalyzed by the NAPE-phospholipase D (NAPE-PLD), producing anandamide (AEA). In addition, phosphatidylinositol (PI) is hydrolyzed by phospholipase C (PLC), originating diacylglycerol (DAG), which is converted to 2-arachidonoylglycerol (2-AG) by diacylglycerol lipase (DAGL). The endocannabinoids AEA and 2-AG act by binding to both type-1 or type-2 cannabinoid receptors (CB1 or CB2). The degradation of the endocannabinoids is mediated by fatty acid amide hydrolase (FAAH), which is responsible for AEA degradation, and by monoacylglycerol lipase (MAGL), which is responsible for 2-AG degradation. The metabolites of AEA and 2-AG are the arachidonic acid (AA) and ethanolamine, and AA and glycerol, respectively. The second panel (*b*) shows the main effects of the endocannabinoids in different tissues. The third panel (*c*) describes the ECS markers in adult rats that were nicotine-exposed during breastfeeding. OMP: osmotic minipumps.

demonstrated that NIC males showed low TSH, T_3 and T_4 plasma levels, while NIC females had higher T_3 and T_4.[12] Here, NIC males did not show changes in thyroid ECS markers, but NIC females had higher thyroid FAAH protein expression. We can speculate that the overexpression of FAAH may avoid the accumulation of AEA and prevent its inhibitory effect on thyroid hormones secretion in females.

Regarding sex dimorphism, there is evidence in the literature that the ECS show sex-related differences. For instance, females seem to have lower activation of ECS than males.[46,64] Considering other programming models, studies reported differences in ECS between sexes.[50,65-67] Here, comparing male and female offspring, the differences observed in ECS markers could have the involvement of the sex hormones. We have already assessed estrogen and testosterone plasma levels in the neonatal nicotine exposure model: NIC females showed no alterations in estradiol and testosterone levels, while NIC males had lower testosterone without changes in estradiol.[12] In the present study, only NIC females were affected. Thus, the protective effect mediated by estrogens [68] did not occur regarding the ECS markers. We have previously detected, in a different experimental model, that adult female rats that were programmed by maternal cigarette smoke during lactation showed lower estradiol levels, higher hypothalamic DAGL protein expression, lower hepatic DALG and lower VAT CB1.[69] Thus, the late effects of nicotine exposure during lactation in the ECS of the female progeny differ from the effects

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observed in females exposed to cigarette smoke for the same period, suggesting that other compounds in the cigarette can also affect ECS tonus. However, in both models (nicotine and smoke), nicotine appears to be responsible for the reduction of DAGL in the liver. We summarize in Fig. 7, the ECS biosynthesis and degradation pathways, the main effects of the endocannabinoids in different tissues and the ECS markers in our experimental model.

Constitute limitations of our study the lack of measurement of AEA and 2-AG levels and of the activity of the enzymes involved in endocannabinoids metabolism.

In conclusion, we demonstrated that nicotine exposure during lactation imprinted a deregulated ECS in the female offspring. The central and peripheral characterization of ECS is a contribution to an understanding of the phenotype observed in this experimental model. Unraveling the associated mechanisms can contribute to the discovery of new pharmacological targets for the treatment of several metabolic diseases originated during development, especially those caused by maternal smoking or caused by the use of nicotine patch by mothers who wish to quit smoking.

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Conflicts of interest. The authors declare that no conflict of interest.

Ethical standards. All experimental procedures performed in this work are in accordance with the ethical standards of the relevant guides on the care and use of laboratory animals and were approved by the institutional ethics committee of the Institute of Biology of the University of the State of Rio de Janeiro (CEUA/033/2017).

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