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Thamara Cherem Peixoto

Avaliação da termogênese e inflamação hipotalâmica em modelos de programação para obesidade

Rio de Janeiro

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-graduação em Biociências da Universidade do Estado do Rio de Janeiro.

Orientadora: Prof.ª Dra. Patrícia Cristina Lisbôa da Silva

Coorientador: Prof. Dr. Egberto Gaspar de Moura

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Rio de Janeiro

2020

DEDICATÓRIA

Aos meus pais (Luiz e Maria Helena), por todos os ensinamentos e valores, por serem meus exemplos de honestidade e determinação. Ao meu marido (Ricardo) por todo companheirismo, amor, compreensão e paciência. As minhas irmãs (Milena e Thays), sem as quais não seria completa. As minhas tias e tio (Alice, Edna, Rosely e Carlos), que estiveram presentes em todas as etapas da minha vida me incentivando. Aos meus orientadores (Patrícia e Egberto) por todo incentivo e dedicação.

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A educação não transforma o mundo. A educação muda pessoas. Pessoas transformam o mundo.

Paulo Freire

RESUMO

PEIXOTO, Thamara Cherem. *Avaliação da termogênese e inflamação hipotalâmica em modelos de programação para obesidade*. 2020. 119 f. Tese (Doutorado em Biociências) – Instituto de Biologia Roberto Alcântara Gomes, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

O tabagismo materno e o desmame precoce, são insultos que ocasionam o desenvolvimento de obesidade e alterações endócrino-metabólicos na prole adulta. Na obesidade ocorrem diversas mudanças no hipotálamo e no tecido adiposo marrom (TAM) em resposta à alterações metabólicas presentes nessa condição. Dessa forma, investigamos as repercussões em longo prazo do tabagismo materno e o desmame precoce sobre a capacidade termogênica do TAM e funcionamento do hipotálamo. **Modelo de exposição da prole à fumaça de cigarro durante a lactação**: ratas Wistar lactantes foram separadas em 2 grupos: FUMAÇA (exposição ao cigarro 3R4F/ 4x / 1h, de PN3-PN21) e CONTROLE (exposição ao ar filtrado). A prole foi eutanasiada em PN180. A exposição neonatal à fumaça do cigarro, em ambos os sexos, reduziu a capacidade termogênica do TAM e alterou a circuitaria hipotalâmica favorecendo a hiperfagia. No entanto, apenas os machos apresentam inflamação no hipotálamo. **Modelo de exposição exclusiva à nicotina durante a lactação:** Em PN3, minibombas foram implantadas em ratas lactantes, que foram divididas em 2 grupos: NICOTINA (6 mg/kg/dia, durante 14 dias) e CONTROLE (solução salina). As proles foram eutanasiados em PN120 e PN180. Evidenciamos que a exposição neonatal à nicotina comprometeu a função do TAM e do tecido adiposo branco em ambos os sexos, contribuindo para suscetibilidade e/manutenção da obesidade nos machos. **Modelo de desmame precoce (DP):** Ratas lactantes foram separadas em 3 grupos: DP NÃO FARMACOLÓGICO: mães foram envolvidas com uma bandagem adesiva nos 3 últimos dias de lactação; DP FARMACOLÓGICO: mães foram tratadas com bromocriptina para bloquear a produção de leite (1mg/Kg PC/dia) nos 3 últimos dias da lactação; CONTROLE: filhotes foram livremente amamentados e desmamados no período padrão (21 dias de vida). Os animais foram eutanasiados em PN180. O desmame precoce alterou a capacidade termogênica do TAM contribuindo parcialmente para a obesidade na idade adulta, apesar de ocorrer diferenças relacionadas ao tipo de desmame. O conjunto dos nossos resultados mostram que o fenótipo de obesidade programada tem relação com a disfunção do TAM e que existem importantes diferenças relacionadas ao sexo nos modelos estudados.

Palavras-chave: Tabagismo materno. Desmame precoce. Programação. Tecido adiposo marrom. Termogênese. Capacidade termogênica. Inflamação hipotalâmica

ABSTRACT

PEIXOTO, Thamara Cherem. *Evaluation of thermogenesis and hypothalamic inflammation in programming models for obesity*. 2020. 119 f. Tese (Doutorado em Biociências) – Instituto de Biologia Roberto Alcântara Gomes, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

Both maternal smoking and early weaning are insults that cause the development of obesity and endocrine-metabolic changes in adult offspring. Several changes occur in the hypothalamus and brown adipose tissue (BAT) in response to obesity. Thus, we investigated the long-term repercussions of maternal smoking and early weaning on the BAT thermogenic capacity and the hypothalamus funtion. **Model of neonatal exposure to cigarette smoke during lactation**: lactating Wistar rats with their pups were separated into 2 groups: SMOKE (exposure to cigarette 3R4F / 4x / 1 h, from PN3-PN21) and CONTROL (exposure to filtered air). The offspring were euthanized in PN180. Direct and indirect exposure to cigarette smoke during lactation reduced the thermogenic capacity of TAM in the offspring from both sexes as well as altered the hypothalamic circuitry favoring hyperphagia. However, only the males showed inflammation in the hypothalamus. **Model of exclusive maternal exposure to nicotine during lactation**: In PN3, mini-pumps were implanted in lactating rats, which were divided into 2 groups: NICOTINE (6 mg / kg BW / day, for 14 days) and CONTROL (saline). The offspring were euthanized in PN120 and PN180. We evidence that postnatal exposure to nicotine compromised the function of TAM and white adipose tissue in both sexes, contributing to the susceptibility and / maintenance of obesity in male offspring. Early weaning (EW) model: lactating rats were randomly separated into 3 groups: NON-PHARMACOLOGICAL EW: mothers were wrapped with an adhesive bandage in the last 3 days of lactation; PHARMACOLOGICAL EW: mothers were treated with bromocriptine to block milk production (1mg / kg BW / day) in the last 3 days of lactation; CONTROL: pups were freely breastfed and weaned in the standard period (21 days of life). The animals were euthanized in PN180. Early weaning altered the thermogenic capacity of BAT, partially contributing to obesity in adulthood, despite differences related to the type of weaning. In summary, we show that the programmed obesity phenotype is related to BAT dysfunction and that there are important sex-related differences in the models studied.

Keywords: Maternal smoking. Early weaning. Programming. Brown adipose tissue. Thermogenesis. Thermogenic capacity. Hypothalamic inflammation.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

11βHSD-1	11-beta-hidroxiesteróide desidrogenase tipo 1
AMPK	Proteína quinase ativada por AMP
AgRP	Proteína relacionada a agouti
ARC	Núcleo arqueado
BRO	Bromocriptina
CB1r	Receptor canabinóide do tipo 1
CPT1a	Carnitina palmitoil transferase 1
CART	Transcrito relacionado à cocaína e à anfetamina
D2R	Receptor dopaminérgico tipo II
DAGL	Diacilglicerol lipase
DAT	Transportador de dopamina
DM2	Diabetes mellitus tipo 2
DNA	Ácido desoxirribonucleico
DP	Desmame precoce
FAS	Ácido graxo sintase
LH	Hipotálamo lateral
MC2R	Receptor de melanocortina do tipo 2
αMSH	Hormônio estimulador de melanócitos α
NPY	Neuropeptídeo Y
OMS	Organização Mundial da Saúde
PFC	Córtex pré-frontal
POMC	Pró-ópiomelanocortina
PGC-1α	Receptor γ ativado por proliferador coativador 1α
PPARγ	Receptor ativado por poliferador de peroxissoma gama
RNA	Ácido ribonucleico
T3	Tritiodotironina
T4	Tiroxina
TA	Tecido adiposo
TAB	Tecido adiposo branco

TAM	Tecido adiposo marrom
TH	Tirosina hidroxilase
TSH	Hormônio estimulante da tireóide
UCP-1	Proteína desacopladora tipo 1
VIGITEL	Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico
VTA	Área tegumental ventral
WHO	World Health Organization

LISTA DE SÍMBOLOS

%	Porcentagem
mg	Miligrama
kg	Kilograma
g	Grama
cm	Centímetro

SUMÁRIO

INTRODUÇÃO

Obesidade

Conceito e epidemiologia

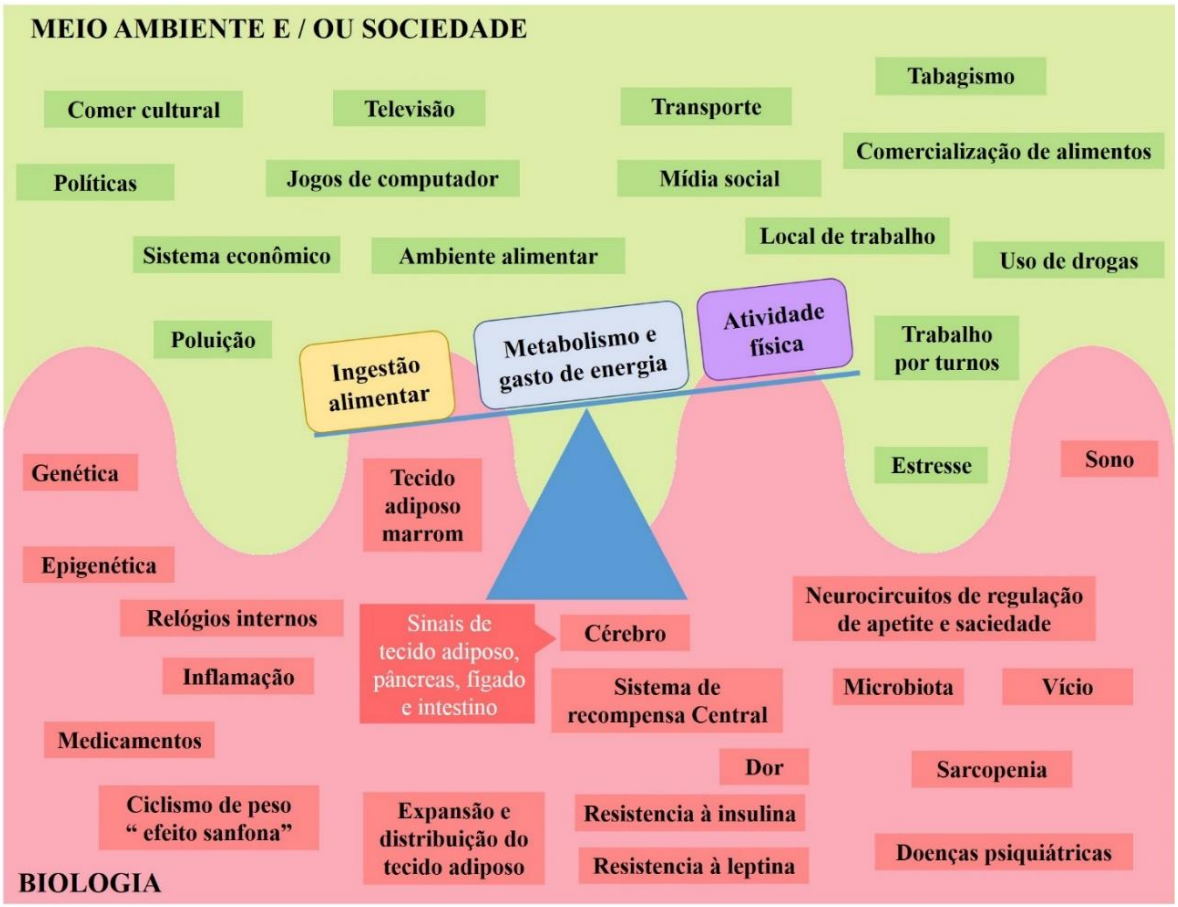
Segundo a Organização Mundial da Saúde (OMS, em inglês *World Health Organization* - WHO), a obesidade pode ser definida como um agravo de caráter multifatorial decorrente de balanço energético positivo que favorece o acúmulo de gordura anormal ou excessivo que apresenta risco à saúde (WHO, 2020a). Acrescentando a definição convencional, a obesidade é uma condição complexa, com sérias dimensões sociais e psicológicas, que afeta praticamente todas as faixas etárias e grupos socioeconômicos (HOFMANN, 2015; RAND et al., 2017).

A obesidade é um dos maiores problemas de saúde pública, uma doença crônica cujo avanço tem se dado de forma acelerada em todo o mundo nos últimos anos (WHO, 2020a). A sua prevalência aumentou em todo o mundo nos últimos 40 anos, atingindo níveis de pandemia. Entre 1975 e 2016, obesidade mundial quase triplicou, em 2016 mais de 1,9 bilhões (39%) da população adulta estava acima do peso, dentre esses 650 milhões (13%) eram obesos. Em relação as crianças e adolescentes, o sobrepeso e obesidade atingiu cerca de 340 milhões. A estimativa é que em 2025, cerca de 2,3 bilhões de adultos estejam com sobrepeso e mais de 700 milhões com obesidade (ABARCA-GÓMEZ, 2017; WHO, 2020a). No Brasil, os índices de sobrepeso e obesidade refletem os padrões mundiais. De acordo com a pesquisa realizada em 2018 pela Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico (Vigitel) do Ministério da Saúde, no qual foram coletados dados em 26 capitais brasileiras e no Distrito Federal (total de 52.395 pessoas entrevistadas), a frequência de excesso de peso foi de 55,7%, sendo ligeiramente maior entre homens (57,8% destes) do que entre mulheres (53,9%) e a frequência de adultos obesos foi de 19,8%, sendo ligeiramente maior entre as mulheres (20,7% destas) do que entre os homens (18,7%) (BRASIL, 2019).

As mudanças no sistema alimentar global caracterizado pelo aumento do consumo de alimentos ultraprocessados, energeticamente densos, ricos em açúcares, gorduras e sódio,

juntamente com o aumento do comportamento sedentário, parecem ser as principais causas impulsionadoras desta doença (BLÜHER et al.,2019). No entanto, existe uma rede complexa de fatores biológicos, ambientais, sociais, genéticos e epigenéticos envolvidos potencialmente na suscetibilidade, desenvolvimento e manutenção da obesidade (BLÜHER et al.,2019; LORENZO et al., 2020) (Figura 1).

Figura 1 – Diferentes fatores envolvidos na patogênese da obesidade



Nota: Fatores biológicos, ambientais e sociais que contribuem para obesidade.
Fonte: Figura adaptada de BLÜHER et al.,2019.

Características da obesidade

A obesidade é uma das doenças crônicas não transmissíveis (DCNT) que epidemiologicamente mais crescem mundialmente (CARNEIRO et al., 2016). É uma doença que acarreta impacto na saúde e qualidade de vida do indivíduo, pois está associada a efeitos

psicológicos negativos (depressão, baixa autoestima e exclusão social) (CAREY et al., 2014; CHU et al., 2019; FLOODY et al., 2018) e a uma variedade de distúrbios metabólicos, tais como dislipidemia, hipertensão arterial, coronariopatias, diabetes mellitus do tipo 2 (DM2), inflamação, problemas respiratórios, apneia do sono, osteoartrite e alguns tipos de câncer (BELYAVSKIY; PIESKE-KRAIGHER; TADIC, 2019; KULKARNI et al., 2016; ENGIN et al, 2017; PATERNOSTER; FALASCA, 2020).

As alterações no estilo de vida representam grande contribuição no desenvolvimento de sobrepeso e obesidade, uma vez que estão diretamente associados com consumo alimentar e gasto energético (CARNEIRO et al., 2016). O tecido adiposo (TA) é um importante órgão envolvido no armazenamento e gasto de energia, relacionado à regulação da homeostase energética (COHEN; SPIEGELMAN, 2015; THOMOU et al., 2017). O TA pode ser classificado devido a sua função, localização e estrutura. Assim, encontramos dois tipos diferentes de tecido adiposo: o tecido adiposo branco (TAB) ou unilocular e o tecido adiposo marrom (TAM) ou multilocular (DENG et al., 2016).

O TAB é caracterizado por armazenar nutrientes sob a forma de triglicerídeos em seu citoplasma como uma grande gotícula de lipídio em seu interior. Ele também é considerado um importante órgão endócrino, sendo utilizado como sensor do estado metabólico, uma vez que é responsável por sintetizar e liberar uma grande quantidade de substâncias peptídicas e não peptídicas como adipocinas, citocinas e quimiocinas (RONTI et al., 2006). O TAM é um tecido multilocular e sua principal função é a termogênese, ou seja, regular a produção de calor e temperatura corporal. Sua cor é proveniente do grande número de mitocôndrias presentes em seu citoplasma (DENG et al., 2016). O mecanismo de dissipar energia pelo TAM envolve a proteína desacopladora 1 (UCP1) presente na membrana interna da mitocôndria e resulta em aumento da oxidação de ácidos graxos e produção de calor (JUNG; SANCHEZ-GURMACHES; GUERTIN, 2018; KALINOVICH et al., 2017).

O TA atualmente é um dos principais focos das pesquisas em obesidade devido sua importante função biológica. Na obesidade ocorrem mudanças funcionais, morfológicas e moleculares nos dois tipos de TA em resposta à alterações metabólicas presentes nessa condição (DENG et al., 2016; TRAYHURN, 2017). O TAB pode sofrer alterações relacionadas ao tamanho do adipócito (hipertrofia) e quantidade de adipócito (hiperplasia). As modificações no tamanho ou no número de adipócitos ocorrem em resposta à ativação de ações metabólicas que promovem adipogênese e lipogênese (QUEIROZ et al., 2009; SMITH; KAHN, 2016). Assim como o TAB, o TAM é um órgão endócrino ativo em adultos. No geral indivíduo obesos apresentam a atividade termogênica reduzida favorecendo a manutenção da

obesidade (ORAVA et al., 2013; SOLER-VÁZQUEZ et al., 2018; VIJGEN et al., 2011). Estudos demonstraram que intensificação e / ou restauração da capacidade termogênica no TAM representa um mecanismo potencial no tratamento da obesidade (LUIJTEN et al., 2019; SAITO et al., 2013; SOLER-VÁZQUEZ et al., 2018). Portanto, ambos os depósitos de TA influenciam o metabolismo, não sendo apenas um componente importante na manutenção do peso corporal, mas também regulando a massa e a composição corporal, além de estar envolvido na patogênese de diversas doenças (DENG et al., 2016; TRAYHURN, 2017).

Já é conhecido que a obesidade está relacionada ao processo de inflamação crônica que compromete o funcionamento normal do organismo. Evidências crescentes sugerem que, assim como nos tecidos periféricos, vias inflamatórias no hipotálamo também são ativadas (VALDEARCOS; XU; KOLIWAD, 2015). Assim, a inflamação hipotalâmica está intrinsecamente associada à patogênese da obesidade, diabetes e suas consequências disfuncionais (SEONG et al., 2019). Por ser o primeiro local envolvido com a sensibilidade dos sinais nutricionais circulantes a inflamação hipotalâmica precede a inflamação sistêmica, onde apenas 1 dia de dieta rica em gordura é capaz de induzir a reatividade de células não neurais, como astrócitos e microglia, gerando, consequentemente, inflamação hipotalâmica (WAISE et al., 2015, SOUZA et al, 2005). É importante ressaltar, que essa inflamação do hipotálamo contribui para resistência a leptina e insulina, favorecendo o ganho de peso e mantendo o peso corporal elevado (SAMODIEN et al., 2019). Considerando a alta prevalência de obesidade na população mundial, há uma necessidade de compreender melhor como esse processo está envolvido nos mecanismos fisiopatológicos da obesidade.

Programação, Obesidade Programada e Modelos Experimentais

Acreditava-se que o material genético era uma espécie de guia definitivo, que determinava todas as características de um indivíduo, desde a cor de seus olhos até sua propensão a doenças (WITTE et al., 1997). No entanto, no início da década de 90, O epidemiologista inglês David Barker já acreditava na influência de condições adversas em períodos de grande plasticidade como, gestação e lactação, sobre a expressão gênica, determinando um padrão de saúde-doença (BARKER, 1995; HALES; BARKER, 1992). Para tentar explicar a relação entre o ambiente fetal e as doenças na vida adulta, como a obesidade, Barker propôs a “hipótese do fenótipo poupador” (HALES; BARKER, 1992). De acordo com

essa teoria, na condição de subnutrição materna, o feto é capaz de se adaptar a um ambiente intra-uterino adverso otimizando o uso de suprimentos energéticos para garantir sua sobrevivência, priorizando o desenvolvimento de órgãos críticos, restringindo os nutrientes para órgãos menos críticos. Basicamente, o que Barker postulou foi que o material genético poderia ser reprogramado, em fases precoces da vida, a fim de adaptar às condições ambientais (HALES; BARKER, 2001).

De acordo com o conceito atual das origens do desenvolvimento da saúde e da doença (em inglês: *Developmental Origins of Health and Disease* - DOHaD), estímulos diversos durante períodos críticos do desenvolvimento podem levar o indivíduo a doenças metabólicas tardias. Se esse indivíduo permanece na condição inicial ele pode se tornar melhor adaptado a sua nova expressão gênica, caso a condição inicial se altere, como por exemplo o indivíduo que sofreu desnutrição intrauterina e, posteriormente, cresce em condições nutricionais de grande oferta energética, esse indivíduo se torna menos adaptado a sua expressão gênica e acaba gerando distúrbios metabólicos (GLUCKMAN; HANSON; 2008; GLUCKMAN et al., 2009; MANDY; NYIRENDA, 2018; SILVEIRA et al., 2007). Assim, o conceito de programação metabólica pode ser definido como a resposta a qualquer evento (ambiental, nutricional e hormonal) durante períodos críticos do desenvolvimento, como os primeiros 1000 dias de vida: gestação (270 dias) + 1° Ano (365 dias) + 2° Ano (365 dias) que podem induzir mudanças epigenéticas que irão determinar o padrão de saúde ou doença mais tarde na vida (LINNÉR; ALMGREN, 2019; VILLARES et al., 2018).

O termo “epigenética” tem origem do grego, onde “epi” significa “em cima de / além de”, e estuda alterações na expressão gênica, sem alterar as sequências de bases nitrogenadas (adenina, guanina, citosina e timina) da molécula de ácido desoxirribonucleico (DNA). A expressão gênica é responsável por direcionar aos caminhos diferentes daqueles diretamente atribuível à sequência de DNA, o que resultará em diferentes fenótipos. As alterações epigenéticas podem ser provocadas pela metilação na molécula de DNA, modificações de histonas, remodelação da cromatina e alterações não-codificantes do RNA. Vale a pena ressaltar que essas alterações podem ser transmitidas à geração seguinte (CAMPIÓN et al., 2010, ROHDE et al., 2019; OBRI et al., 2020).

Alterações epigenéticas nos primeiros mil dias de vida podem contribuir para o desenvolvimento de distúrbios metabólicos na idade adulta, sendo denominada de programação metabólica (WATERLAND; GARZA, 1999; LINNÉR; ALMGREN, 2019). Atualmente, diversos estudos apontam os potenciais mecanismos envolvidos na programação, principalmente associado ao desenvolvimento de obesidade no adulto (GADDE et al., 2018;

OBRI et al., 2020; OESTREICH et al., 2017; ROHDE et al., 2019). A condição de nutrição materna em relação a qualidade ou quantidade de nutrientes consumidos durante a gravidez pode exercer efeitos permanentes e poderosos sobre o desenvolvimento da prole, aumentando a predisposição para o desenvolvimento de distúrbios metabólicos (ELSHENAWY et al., 2016; GODFREY et al., 2017; LANGLEY-EVANS, 2014; NAVARRO, 2017). Por outro lado, a fase da lactação também é considerada um período crítico que pode programar a prole. Há cerca de 20 anos, o nosso grupo de pesquisa tem estudado a programação neonatal da obesidade causada por insultos impostos exclusivamente durante o período de amamentação, concentrando os estudos na avalição de mecanismos fisiopatológicos pelos quais fatores nutricionais, ambientais e hormonais atuam durante a lactação determinando alterações futuras quanto a maior adiposidade, alterações na ingestão alimentar e diversos distúrbios endócrino-metabólicos (MOURA; LISBOA; PASSOS, 2008).

A exposição ao cigarro durante a lactação é um insulto que pode programar à obesidade tardia e suas complicações metabólicas nos descendentes (LISBOA et al., 2017; SANTOS-SILVA et al., 2013). A nutrição da criança nos primeiros dois anos de vida é determinante para as origens das doenças no desenvolvimento (VILLARES et al., 2018), sendo o desmame precoce também um fator de programação metabólica (LIMA et al., 2013; PIETROBON et al., 2019; SOUZA; MOURA; LISBOA, 2020). Dentre os modelos animais de programação estudados em nosso laboratório, destacamos os que serão abordados neste trabalho: 1) modelos de tabagismo materno: exposição exclusiva à nicotina durante a lactação (OLIVEIRA et al., 2009, 2010a; PINHEIRO et al., 2011) e exposição direta e indireta da prole à fumaça de cigarro durante a lactação (LISBOA et al., 2017; SANTOS-SILVA et al., 2013); 2) modelos de desmame precoce: farmacológico (BONOMO et al., 2007; MOURA et al., 2009) e não farmacológico (LIMA et al., 2013).

Modelo de programação pela exposição exclusiva à nicotina durante a lactação

O tabagismo é considerado uma epidemia e passou a ser classificado como uma doença crônica causada pela dependência química dos fumantes à nicotina. Aproximadamente, 5,4 milhões de pessoas morrem todos os anos de doenças relacionadas ao tabaco (WHO, 2019). A menos que uma ação urgente seja tomada, estima-se que em 2030 haverá mais de 8 milhões de mortes relacionadas ao tabaco (WHO, 2019). Segundo dados

recentes da VIGITEL, no Brasil, a frequência de adultos fumantes em 2018 foi de 9,3%, sendo a prevalência de 12,1% em homens e 6,9% em mulheres (BRASIL, 2019).

O baixo custo para o consumidor e a falta de consciência sobre os efeitos deletérios para saúde tornam o cigarro um produto acessível e altamente utilizado em todo o mundo. Assim, o cigarro é um produto de consumo legal que mata mais da metade dos usuários e que tem relação com aproximadamente 50 doenças, entre elas vários tipos de câncer (pulmão, laringe, faringe, esôfago), doenças do aparelho respiratório (enfisema pulmonar, bronquite crônica, asma, infecções respiratórias), doenças cardiovasculares (angina, infarto agudo do miocárdio, hipertensão arterial) e acidente vascular cerebral (INSTITUTO NACIONAL DE CÂNCER, 2018a). É importante ressaltar que os fumantes passivos, ou seja, aqueles expostos à fumaça do cigarro de maneira involuntária, também apresentam prejuízos à saúde, podendo desenvolver reações alérgicas, infarto agudo do miocárdio, câncer e doenças respiratórias (INSTITUTO NACIONAL DO CÂNCER, 2018b).

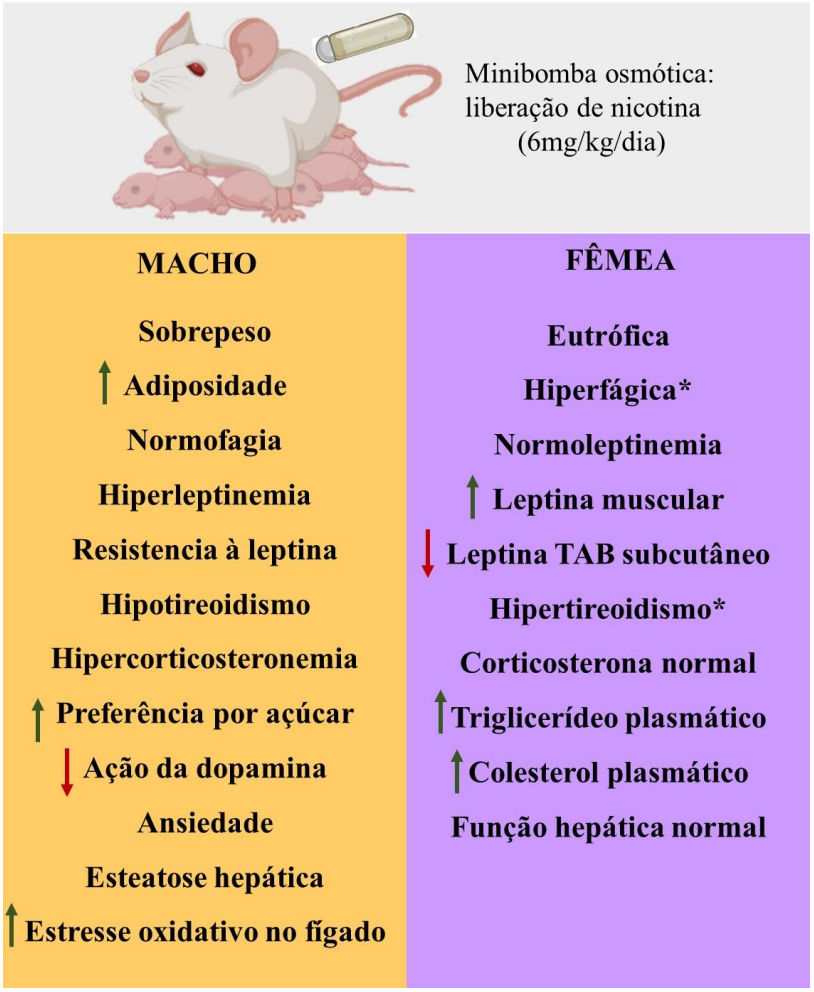
O cigarro contém mais de 7.000 compostos (WHO, 2019), sendo a nicotina considerada o principal composto psicoativo capaz de causar dependência (RODGMAN; PERFETTI; 2013). A manutenção da dependência da nicotina está associada à sensação de prazer causada pelo seu consumo e aos efeitos adversos da abstinência que reforça o comportamento de autoadministração (LEWIS; MILLER; LEA, 2007). A nicotina é um importante poluente ambiental, capaz de se propagar para o solo e atmosfera (SELMAR et al., 2018). Muitas evidências indicam que a nicotina é capaz de atuar como disruptor endócrino em fases críticas do desenvolvimento, acarretando alterações epigenéticas que induzem disfunções metabólicas (MIRANDA; MOURA; LISBOA, 2020).

Sabe-se que aproximadamente 9% a 20% das mulheres relatam fumar durante a gestação, sendo o cigarro a droga mais utilizada por gestantes (AL-SAHAB et al., 2010; MEERNIK; GOLDSTEIN, 2015; NAPIERALA et al., 2016). O tabagismo materno está associado a desfechos fetais, obstétricos e de desenvolvimento adversos (ABRAHAM et al., 2017; BERGMANN et al., 2008; JAKAB, 2010). Conhecendo os efeitos nocivos do cigarro muitas mulheres pararam de fumar durante a gestação, mas retornam durante a lactação, acreditando que o uso do cigarro nesse período não irá interferir na saúde do seu filho (GIBBS; COLLACO; MCGRATH-MORROW, 2016; HANNÖVER et al., 2008). No entanto, a nicotina é transferida pelo leite materno e é capaz de promover vários efeitos adversos de curto e longo prazos (NAPIERALA et al., 2016; GIBBS; COLLACO; MCGRATH-MORROW, 2016; OLIVEIRA et al., 2009, 2010a).

Nesse sentido, para mimetizar o uso de cigarro durante a lactação pautando seus efeitos exclusivamente sobre a nicotina, nosso laboratório desenvolveu o modelo animal de programação pela exposição exclusiva à nicotina durante a lactação, para compreender como a exposição desse composto via leite materno, poderia afetar o padrão de "saúde e doença" na prole adulta. Neste modelo experimental, a exposição exclusiva à nicotina ocorre através de minibombas osmóticas do 2° ao 16° dia da lactação. As ratas lactantes são anestesiadas para uma pequena incisão no dorso (3x6 cm) para inserção das minibombas osmóticas que liberam na corrente sanguínea 6mg/kg de nicotina por dia durante os 14 dias consecutivos. Esta dose utilizada é equivalente à usada por fumantes pesados. Foi observado a presença de cotinina, o principal metabólito da nicotina, no leite das ratas e no plasma dos filhotes expostos à nicotina, comprovando a eficácia do modelo (OLIVEIRA et al., 2010a).

Os machos adultos programados pela exposição exclusiva da nicotina durante a lactação apresentam aumento de peso e gordura corporal, hiperleptinemia (OLIVEIRA et al., 2009; SANTOS-SILVA et al., 2011), menor sinalização da leptina no hipotálamo (OLIVEIRA et al., 2010b) hipotireoidismo secundário (OLIVEIRA et al., 2009; YOUNES-RAPOSO et al., 2013; LISBOA et al., 2015), hipercorticosteronemia (PINHEIRO et al., 2011), esteatose hepática (BERTASSO et al., 2020a; CONCEIÇÃO et al., 2015), preferência por dieta rica em açúcar, menor ação da dopamina, maior ansiedade (PINHEIRO et al., 2015a) e inflamação hipotalâmica (YOUNES-RAPOSO et al., 2015a). O que é conhecido das fêmeas adultas deste modelo, é que são eutróficas, não apresentam alteração de leptina e corticosterona plasmática, apresentam menor leptina no tecido adiposo subcutâneo e maior leptina no músculo (PINHEIRO et al., 2011), apresentam aumento de triglicerídeo e colesterol no plasma (BERTASSO et al., 2020a). A Figura 2 aponta os principais resultados do modelo de programação pela exposição exclusiva à nicotina durante a lactação na prole adulta de ambos os sexos.

Figura 2 – Principais resultados do modelo de programação pela exposição exclusiva à nicotina durante a lactação (prole adulta)



Legenda: Tecido adiposo branco (TAB); ***** dados não publicados.
Fonte: A autora, 2020.

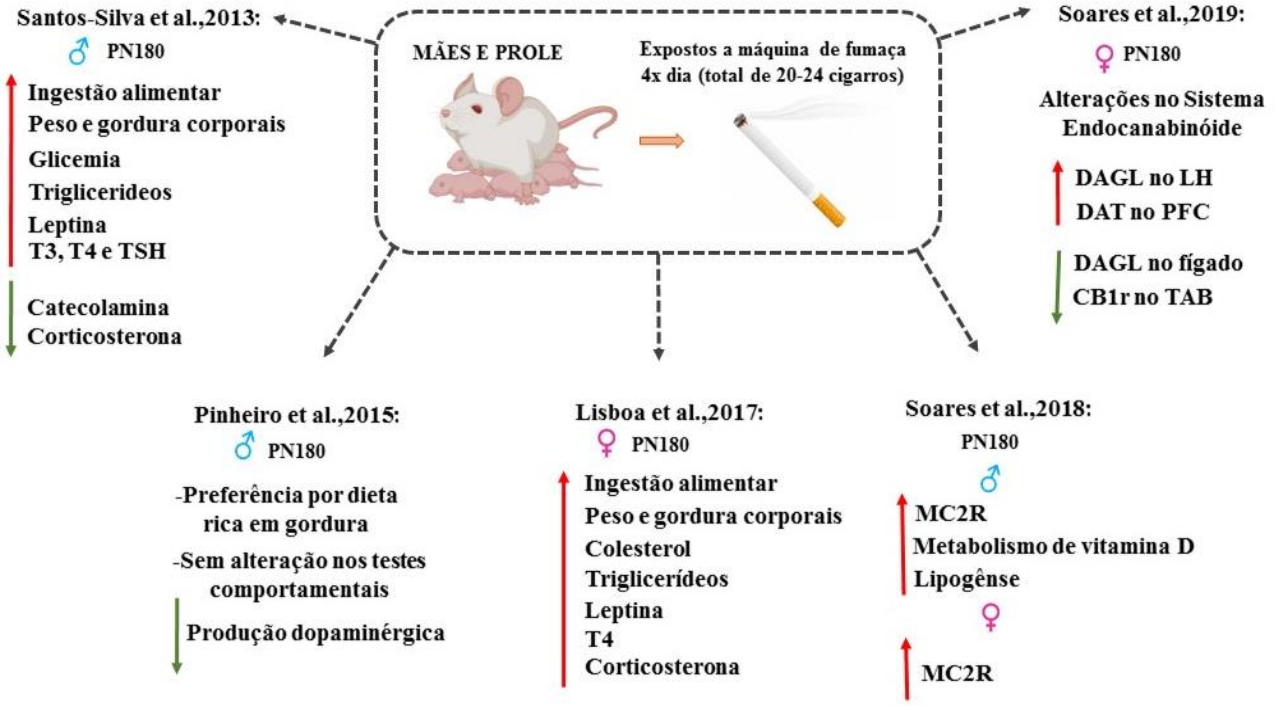
Modelo de programação pela exposição direta e indireta da prole à fumaça de cigarro durante a lactação

Como já relatado anteriormente, muitas mulheres fumam durante a gestação e lactação. Alguns estudos indicam que o uso de cigarro durante essas fases críticas do desenvolvimento, pode comprometer o desenvolvimento da criança à curto e longo prazo, podendo aumentar o risco desses indivíduos se tornarem obesos e diabéticos quando adultos (MONTGOMERY; EKBOM, 2002; OLIVEIRA et al.,2009; OKEN; LEVITAN; GILLMAN, 2008). Nesse sentindo, para melhor compreender os impactos do uso de cigarro apenas durante o período da lactação, nosso laboratório desenvolveu o modelo animal de programação pela exposição direta e indireta da prole à fumaça de cigarro durante a lactação.

Neste modelo experimental, as mães e filhotes são expostos a fumaça do cigarro do dia 3° ao 21° dia da lactação. Os animais são alojados diariamente numa máquina de fumaça por 4 períodos (1 h cada exposição). Durante essa 1 h, a máquina queima de 5 a 6 cigarros de pesquisa 3R4F, contendo 0,73 mg nicotina cada (SANTOS-SILVA et al., 2013). Esta dose utilizada é equivalente à usada por fumantes moderados à pesados. Neste modelo também foi observado a presença de cotinina no leite das ratas e no plasma dos filhotes expostos à fumaça do cigarro, comprovando a eficácia do modelo (SANTOS-SILVA et al., 2011).

Quando adultos, os animais de ambos os sexos foram programados para aumento de ingestão alimentar, peso e gordura corporal, triglicerídeos, leptina plasmática e do conteúdo de receptor de melanocortina do tipo 2 (MC2R) na adrenal (SANTOS-SILVA et al., 2013; LISBOA et al., 2017; SOARES et al., 2018). Os machos apresentam também aumento de glicemia, T3, T4 e TSH, redução de corticosterona e do conteúdo de catecolamina adrenal (SANTOS-SILVA et al., 2013), preferência por dieta rica em gordura, alterações no sistema dopaminérgico (PINHEIRO et al., 2015), aumento do potencial lipogênico e do metabolismo de vitamina D no fígado (SOARES et al., 2018). Enquanto as fêmeas apresentam aumento plasmático de colesterol, T4 e corticosterona (LISBOA et al., 2017) e alteração no sistema endocanabinóide (SOARES et al., 2019). A Figura 3 resume os principais resultados do modelo de programação pela exposição direta e indireta da prole à fumaça do cigarro na prole adulta de ambos os sexos.

Figura 3 – Principais resultados do modelo de programação pela exposição direta e indireta da prole à fumaça de cigarro (prole adulta)



Legenda: T3: Triiodotironina (T3); Tiroxina (T4); Tireotrofina (TSH); Tirosina hidroxilase (TH); Área tegumental ventral (VTA); Receptor dopaminérgico tipo II (D2R); Núcleo accumbens (NAc); Receptor de melanocortina do tipo 2 (MC2R); Diacilglicerol lipase (DAGL); Hipotálamo lateral (LH); Transportador de dopamina (DAT); Córtex pré-frontal (PFC); Receptor canabinóide do tipo 1 (CB1r); Tecido adiposo branco (TAB); Núcleo arqueado (ARC).

Fonte: A autora, 2020.

Modelo de programação pelo desmame precoce (farmacológico e não farmacológico)

O aleitamento materno é a primeira prática alimentar recomendada para a promoção da saúde e adequado desenvolvimento infantil, devendo ser complementado a partir dos 6 meses de vida até os 2 anos. A OMS, endossada pelo Ministério da Saúde do Brasil, recomenda o aleitamento materno exclusivo por seis meses e ressalta os benefícios para mãe e bebê (BRASIL, 2015; WHO 2020b). Para mães, os benefícios estão relacionados ao menor sangramento pós-parto, recuperação mais rápida, proteção contra câncer de mama, além da promoção do vínculo afetivo entre mãe e filho. Para o bebê, os benefícios apontados são: proteção contra infecções gastrointestinais e respiratórias, redução da mortalidade na infância, redução do risco de alergias e menor chances de desenvolver obesidade, além do leite ser um alimento completo que apresenta todos nutrientes essenciais para o crescimento e o desenvolvimento saudável (BRASIL, 2015; HORTA; VICTORA, 2013). Ainda nessa perspectiva, o leite materno garante muito mais do que a nutrição da criança, é um alimento natural que gera vínculo, afeto, proteção e nutrição e constitui a mais sensível, econômica e eficaz ferramenta para redução da morbimortalidade infantil (BRASIL, 2015; WHO 2020b).

Apesar de grandes avanços nos índices de aleitamento materno exclusivo desde a década de 80 (BOCCOLINI et al., 2017) e dos inúmeros benefícios apontados, apenas cerca de 36% das crianças são amamentadas dentro dessas recomendações (WHO 2020b). Esses dados mundiais são alarmantes e comprovam que a prática da amamentação exclusiva ainda é muito pequena. No Brasil, a “epidemia do desmame” iniciou na década de 1970, devido ao intenso processo de urbanização, o aumento do número de mulheres que ingressavam no mercado de trabalho e as incansáveis propagandas e publicidades para comercialização do leite industrializado em todo o mundo (VENANCIO; SALDIVA; MONTEIRO, 2013). A II Pesquisa de Prevalência de Aleitamento Materno nas capitais brasileiras e no Distrito Federal, que teve como objetivo verificar a situação da amamentação e da alimentação complementar no Brasil, no período de 1999 a 2008, revelou que apenas 41% das crianças menores de 6 meses estavam em aleitamento materno exclusivo (BRASIL, 2009).

O desmame precoce, ou seja, a privação total ou parcial do leite materno antes que o bebê complete seis meses de vida, ocasiona impactos negativos a curto e longo prazo, tais como sobrepeso e obesidade, além de alterações no perfil lipídico, pressão arterial, e DM2 (BRASIL, 2015, ODDY, 2012; ONIS et al., 2013). Levando em consideração estes fatos, são

necessárias mais ações que promovam, incentivem e apoiem o aleitamento materno exclusivo por seis meses (TAMASIA; SANCHES, 2016).

Estudos experimentais que utilizam o desmame precoce por privação materna demonstram efeitos negativos sobre o metabolismo (aumento da lipogênese hepática e menor sensibilidade a insulina) (MELA et al., 2012; NAKAGAKI et al., 2018) e também sobre o comportamento (aumento de corticosterona, ansiedade e agressividade) (KIKUSUI, 2005, 2006; KIKUSUI; TAKEUCHI; MORI, 2004). Nesse modelo experimental existe dois fatores atuando: a restrição de leite materno e restrição do cuidado materno. Assim, os resultados apresentados podem ser devido a respostas adaptativas às mudanças nutricionais ou ao estresse emocional da separação materna, bem como a associação desses dois fatores. Nesse sentindo, o desenvolvimento de um modelo de desmame precoce sem a separação materna não apresentará fator de confundimento, isolando apenas o efeito da interrupção do aleitamento materno, se aproximando mais das condições humanas que acarretam o desmame precoce.

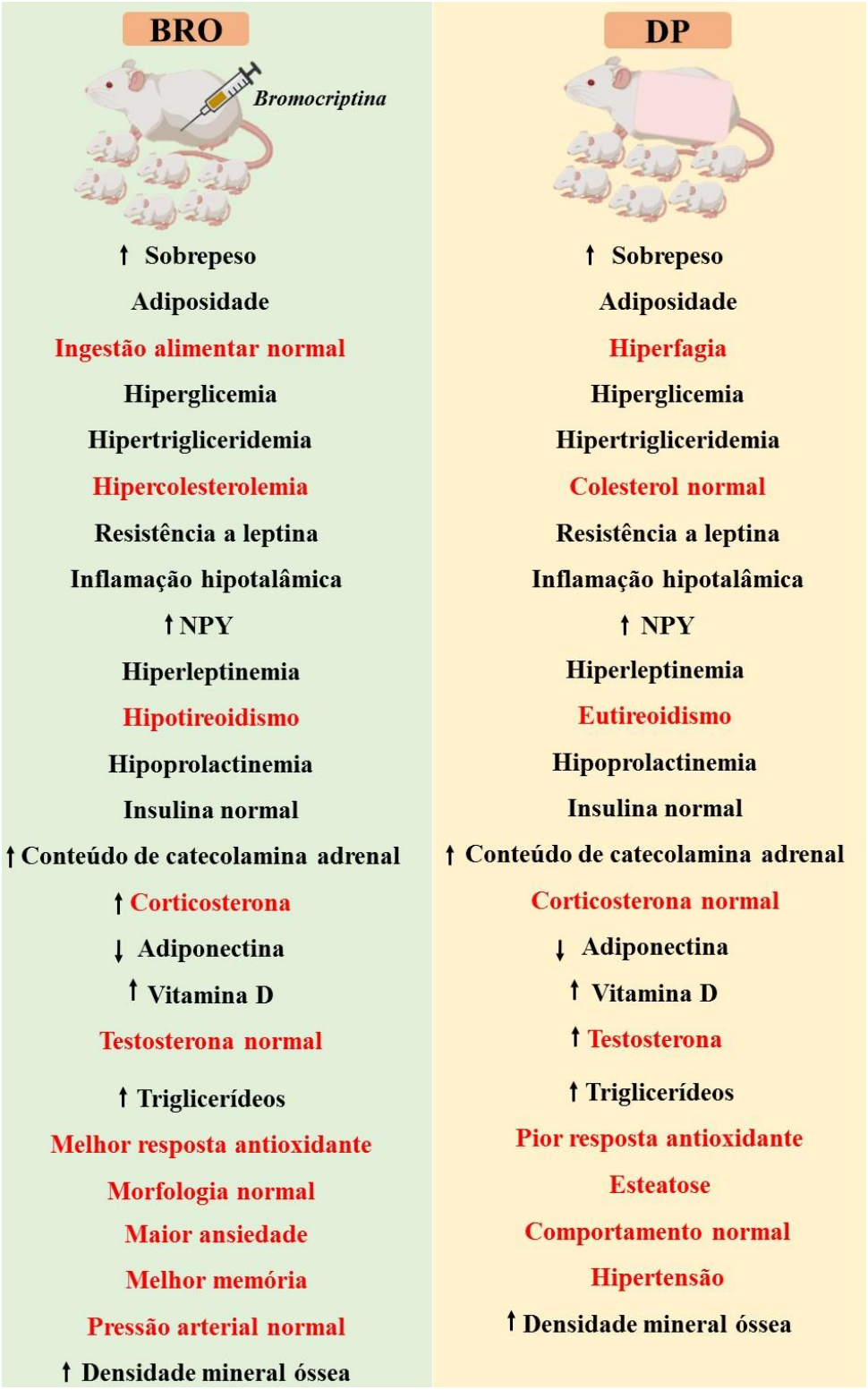
Considerando o cenário de baixa adesão de aleitamento materno exclusivo por seis meses (cerca de 40%), o nosso laboratório desenvolveu dois modelos animais de programação pelo desmame precoce: farmacológico e não farmacológico com o intuito de melhor compreender os impactos negativos da interrupção do aleitamento materno exclusivo antes dos seis meses sobre o desenvolvimento de distúrbios endócrino-metabólicos.

O modelo de desmame precoce farmacológico, o qual é realizado através do tratamento materno com bromocriptina (BRO), um fármaco pertencente à classe dos agonistas dos receptores dopaminérgicos do tipo 2, capaz de atravessar a barreira hematoencefálica e agir nas células lactotróficas da adeno-hipófise inibindo a secreção de prolactina, bloqueando a produção de leite materno (LANDGRAF et al., 1977; FRIIS; PAULSON; HERTZ, 1979). O tratamento com BRO foi realizado nas ratas lactantes nos 3 últimos dias que antecedem o desmame habitual (21 dias) por injeções intraperitoneais de 0.5 mg administradas 2 vezes ao dia (BONOMO et al., 2005). É importante ressaltar que um dia de lactação para um rato é equivalente a 8,6 dias em humanos, então o período de 21 dias de lactação nos ratos corresponde os 6 meses de aleitamento exclusivo em humanos (QUINN, 2005). Assim, comparativamente, o desmame em nosso modelo experimental ocorre cerca de 5 meses. Os resultados anteriores demonstram que os animais desmamados precocemente, de ambos os sexos quando adultos, apresentam aumento de gordura visceral (BONOMO et al., 2007, 2008; PIETROBON; BERTASSO et al., 2019), hipertrofia dos adipócitos (PEIXOTO-SILVA et al., 2014; PIETROBON; BERTASSO et al., 2019) e aumento de corticosterona sérica

(MIRANDA et al., 2019; MOURA et al., 2009). Os machos apresentam ainda hiperleptinemia, resistência à leptina, hipotireoidismo central, dislipidemia (BONOMO et al., 2007; BONOMO et al., 2008; MOURA et al., 2009) e inflamação hipotalâmica (YOUNES-RAPOZO et al., 2015b).

O modelo experimental desmame precoce não farmacológico (DP) consiste na criação de uma barreira física para interromper a amamentação. Para esse procedimento as ratas lactantes são levemente anestesiadas e cuidadosamente enfaixadas com bandagens adesivas, de maneira que todas as tetas fiquem cobertas, privando os filhotes ao leite materno nos 3 últimos dias da lactação. A partir desse período (18° dia de vida pós-natal), os filhotes passam a comer ração padrão (LIMA et al., 2011). Os animais adultos de ambos os sexos apresentaram aumento de gordura corporal, hipertrofia dos adipócitos, hiperfagia e hiperleptinemia (LIMA et al.; 2011; PIETROBON; BERTASSO et al., 2019). Os machos apresentam ainda resistência central à leptina, disfunção da adrenal (LIMA et al., 2011; LIMA et al., 2013), inflamação hipotalâmica (YOUNES-RAPOZO et al., 2015b) e esteatose hepática (BERTASSO et al., 2020b; FRANCO et al., 2013). As Figuras 4 e 5 apontam os principais resultados da programação pelo desmame precoce farmacológico e não farmacológico na prole adulta de ambos os sexos.

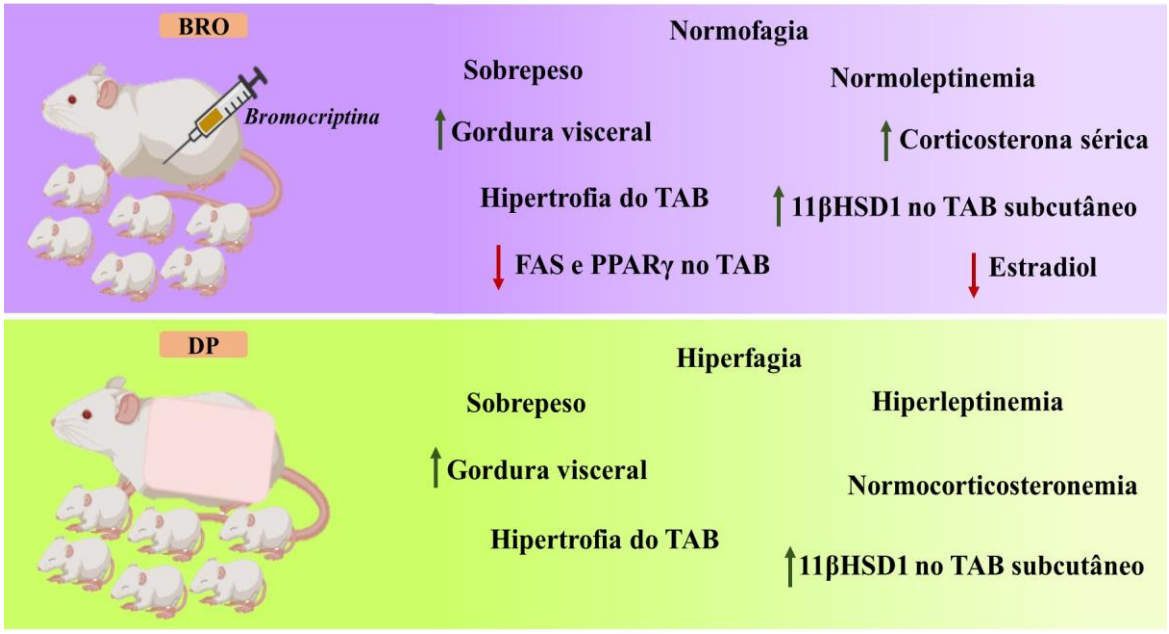
Figura 4 – Principais resultados do modelo de programação pelo desmame precoce (farmacológico e não farmacológico) nos animais adultos do sexo masculino



Legenda: Neuropeptídeo Y (NPY); Modelo de desmame precoce farmacológico utilizando bromocriptina (BRO); Modelo de desmame precoce não farmacológico (DP).

Fonte: Adaptada de SOUZA et al.,2020.

Figura 5 – Principais resultados do modelo de programação pelo desmame precoce (farmacológico e não farmacológico) nos animais adultos do sexo feminino



Legenda: Modelo de desmame precoce farmacológico utilizando bromocriptina (BRO); Modelo de desmame precoce não farmacológico (DP); Tecido adiposo branco (TAB); Ácido graxo sintase (FAS); Receptores ativados por proliferador de peroxissoma gama (PPARγ); 11β-hidroxiesteróide desidrogenase tipo 1 (11βHSD1).
Nota: Esses resultados podem ser encontrados na lista de referências: BERTASSO et al., 2020b; Miranda et al., 2019; PIETROBON; BERTASSO et al., 2019.
Fonte: A autora, 2020.

Termogênese

A termogênese é o processo de produção de energia sobre forma de calor e é um importante mediador do mecanismo de termorregulação da temperatura corporal em mamíferos. Do ponto de vista fisiológico, a termogênese pode ser dividida em duas categorias: termogênese obrigatória e termogênese facultativa ou adaptativa (BIANCO, 2009; CANNON; NEDERGAARD, 2004; CHOUCHANI; KAZAK; SPIEGELMAN, 2019). A termogênese obrigatória é aquela associada a manutenção da taxa metabólica basal, ou seja, é a somatória de todo o calor produzido no organismo em seu estado de vigília e repouso, em temperatura ambiente, em jejum (mínimo 12 horas), capaz de manter funções como, batimentos cardíacos, pressão arterial e respiração (BIANCO, 2009; CANNON; NEDERGAARD, 2004). A termogênese facultativa ou adaptativa, é todo calor produzido além da taxa metabólica basal resultante de estímulos diversos, tais como mudanças de

temperatura, ingestão alimentar e atividade física. Esse tipo de termogênese é subdivida em: com tremor e sem tremor. A termogênese adaptativa com tremor é realizada pelos músculos, onde a contração involuntária das miofibrilas produz calor. Já a termogênese adaptativa sem tremor, também conhecida como clássica, refere-se à produção de energia sobre forma de calor que ocorre através do tecido adiposo marrom (TAM), um órgão especializado e essencial para esse tipo de termogênese (BIANCO, 2009; CANNON; NEDERGAARD, 2004; CHOUCHANI; KAZAK; SPIEGELMAN, 2019).

O TAM, cuja principal função é a termogênese, expressa altos níveis de UCP1, uma proteína chave neste processo que aumenta a permeabilidade da membrana mitocondrial interna, desviando prótons do ciclo oxidativo, levando à dissipação de energia por meio da produção de calor. A função termogênica do TAM depende de muitos fatores diferentes, como temperatura, ingestão de alimentos, atividade física, status metabólico e ação hormonal, sendo sua capacidade termogênica é proporcional à quantidade de UCP1 (JUNG; SANCHEZ-GURMACHES; GUERTIN, 2018; KALINOVICH et al., 2017). O TAM é totalmente inervado por fibras eferentes simpáticas que garantem o controle central da termogênese principalmente via receptores β3-adrenérgicos (CONTRERAS et al., 2014; MORRISON; MADDEN; TUPONE, 2014). Outros mecanismos estão envolvidos nesse controle fino da termogênese sem tremores e algumas moléculas são considerados importantes biomarcadores da capacidade termogênica do TAM, tais como os hormônios tiroidianos que são capazes de aumentar atividade UCP1 e a resposta simpática adrenérgica (MARTÍNEZ-SÁNCHEZ et al., 2014), o receptor γ ativado por proliferador coativador 1α (PGC-1α), que regula a biogênese mitocondrial, o ciclo de Krebs e a beta-oxidação (FERNANDEZ-MARCOS; AUWERX, 2011), a carnitina palmitoil transferase 1 (CPT1a), que catalisa a transformação de ácidos graxos de cadeia longa em acilcarnitinas, etapa essencial para a captação mitocondrial de ácidos graxos de cadeia longa e sua subsequente beta-oxidação (BONNEFONT, 2004) e a proteína quinase ativada por AMP (AMPK), que está relacionada a integridade mitocondrial e regulação da atividade UCP1 (DESJARDINS; STEINBERG, 2018).

Acreditava-se que o TAM era presente e ativo apenas em recém-nascidos, contudo vários estudos vêm demonstrando que humanos adultos apresentam TAM funcional (CYPESS et al., 2009; LICHTENBELT et al., 2009; VIRTANEN et al., 2009). Essa descoberta vem impulsionando pesquisas que buscam compreender o papel do TAM no metabolismo. Estudos demonstraram que intensificação e / ou restauração da termogênese no TAM representa um potencial mecanismo contra a obesidade e distúrbios metabólicos associados, podendo contribuir para o aumento do gasto energético, redução da massa

corporal e melhora do status metabólico (LUIJTEN et al., 2019; SAITO, 2013; SOLER-VÁZQUEZ et al., 2018).

Devido a sua importante função termogênica, o TAM pode ser considerado um alvo terapêutico potencial para melhorar as comorbidades associadas à obesidade. Na obesidade ocorrem mudanças morfofuncionais no TAM (DENG et al., 2016; TRAYHURN, 2017). Geralmente obesos apresentam a atividade reduzida da termogênese, prejudicando o gasto energético e favorecendo a manutenção da obesidade (ORAVA et al., 2013; SOLER-VÁZQUEZ et al., 2018; VIJGEN et al., 2012). Estudos em animais, relataram alteração no funcionamento e estrutura do TAM durante a progressão da obesidade, demonstrando que a administração de uma dieta hiperlipídica é responsável por alterar parâmetros moleculares do TAM de maneira dependente do tempo (MCGREGOR et al., 2013), além de aumentar também o processo inflamatório e estresse oxidativo do tecido (ALCALÁ et al., 2017). Nessa perspectiva, alguns estudos apontam que o transplante de TAM saudável é capaz de melhorar o funcionamento metabólico de animais obesos (WHITE; DEWAL; STANFORD, 2019). No modelo animal de obesidade induzida por dieta rica em gordura, o transplante de TAM foi capaz de melhor tolerância à glicose, aumentar a sensibilidade à insulina, reduzir peso e gordura corporal (SOLER-VÁZQUEZ et al., 2018; STANFORD et al., 2013). Portanto, o TAM influencia no metabolismo, não apenas por sua capacidade termogênica inerente, mas também pela sua capacidade de melhorar o metabolismo da glicose (WHITE; DEWAL; STANFORD, 2019). À medida que as taxas de obesidade crescem em toda população mundial, o desenvolvimento de novas terapias e tratamentos para o combate e prevenção da obesidade tornam-se cada vez mais importantes.

Inflamação hipotalâmica

Muitos anos de pesquisas revelaram que hipotálamo é a principal região do cérebro envolvida na regulação da homeostase energética, em particular, o núcleo arqueado (ARC) do hipotálamo médio basal (DATE et al., 1999; OBICI et al. 2002; SEONG et al., 2019). O hipotálamo está posicionado na base do cérebro, próximo a barreira hematoencefálica, local perfeito para monitorar, processar e transmitir informações para os órgãos periféricos (KIM; CHOE, 2019). A regulação do comportamento alimentar e gasto de energético ocorre por meio de neurônios presentes no ARC, chamados anorexígenos, que liberam neuropeptídios

como pró-ópiomelanocortina (POMC), hormônio estimulador de melanócitos α (αMSH) e o transcrito relacionado à cocaína e à anfetamina (CART), que reduzem a ingestão alimentar e aumentam o gasto de energético, sendo regulados positivamente pela leptina, e por meio de neurônios orexígenos que secretam neuropeptídeos, tais como neuropeptídeo Y (NPY) e proteína relacionada a agouti (AgRP), que aumentam a ingestão alimentar e reduzem o gasto de energético, sendo regulados negativamente pela leptina (VALDEARCOS; XU; KOLIWAD, 2015).

É conhecido que obesidade induz inflamação sistêmica de baixo grau (HOTAMISLIGIL, 2006) e evidências emergentes mostram que o consumo exagerado de gordura induz ativação de células não neuronais como astrócitos e microglia para produzir inflamação hipotalâmica (FRITSCHE, 2015; GUPTA et al. 2012; VALDEARCOS et al. 2017). Thaler et al. 2012, utilizando um modelo animal, demonstraram que o consumo de dieta rica em gordura por 1 a 3 dias é suficiente para induzir a inflamação hipotalâmica sem qualquer ganho de peso visível (THALER et al., 2012). Assim, o comprometimento da atividade hipotalâmica é capaz de alterar circuitos hormonais e neuronais que regulam a homeostase energética, causando obesidade e comorbidades subsequentes (VALDEARCOS; XU; KOLIWAD, 2015).

O processo de inflamação no hipotálamo ocorre em duas fases. Uma fase inicial momentânea, em resposta ao insulto de uma refeição hiperlipídica com intuito de limitar ou reparar a lesão neuronal, e uma fase secundária, devido a exposição crônica à dieta hiperlipídica, onde cascatas inflamatórias levam à ativação de mecanismos de estresse celular, que comprometem a sinalização local da insulina e leptina prejudicado a homeostase energética (JAIS; BRÜNING, 2017). Portanto, a inflamação hipotalâmica induzida por dieta hiperlipídica precede o início da manifestação obesidade e ocorre muito antes da inflamação ou distúrbios metabólicos nos tecidos periféricos (VALDEARCOS; XU; KOLIWAD, 2015; VELLOSO; ARAÚJO; SOUZA, 2008).

Como já relatado, a inflamação hipotalâmica ocorre rapidamente (dentro de 24h) em resposta a uma refeição hiperlipídica (WAISE et al., 2015), no entanto a recuperação da função do hipotálamo é lenta. Um estudo demostrou que, mesmo após 8 semanas de consumo de ração padrão e perda de peso, a inflamação hipotalâmica em resposta a dieta hiperlipídica ainda era evidente (WANG et al., 2012). Evidências recentes deixam claro que o consumo agudo a crônico de dieta rica em gordura causam inflamação hipotalâmica via produção de citocinas pró-inflamatórias em astrócitos e microglia contribuindo para a patogênese da obesidade (SAMODIEN et al., 2019; SEONG et al., 2019). Dessa forma, impedir ou restaurar

o processo de inflamação no hipotálamo pode ser alvo para o desenvolvimento de novas terapias de tratamento para obesidade.

1 **JUSTIFICATIVA**

A obesidade é um dos maiores problemas de saúde pública, cujo avanço tem se dado de forma acelerada em todo o mundo nos últimos anos (CARNEIRO et al., 2016; WHO, 2020a). Diversos estudos apontam os potenciais mecanismos envolvidos para explicar a patogênese da obesidade. Um dos mecanismos moleculares propostos é o papel da epigenética sobre o desenvolvimento da obesidade e suas complicações metabólicas (GADDE et al., 2018; ROHDE et al., 2019). Nesse sentindo, o padrão de saúde ou doença na vida adulta está relacionado a alterações (ambiental e nutricional) que ocorrem durante períodos de grande plasticidade, como gestação e lactação, podendo induzir mudanças epigenéticas permanentes. Diferentes insultos estão envolvidos na programação, aqui destacamos o tabagismo materno (MIRANDA; MOURA; LISBOA, 2020) e o desmame precoce (SOUZA; MOURA; LISBOA, 2020) que ocasionam o desenvolvimento de obesidade e alterações endócrino-metabólicos na prole adulta.

Na obesidade ocorrem diversas mudanças no TAM em resposta alterações metabólicas presentes nessa condição (DENG et al., 2016; TRAYHURN, 2017). Indivíduos obesos apresentam menor atividade termogênica, prejudicando o gasto energético e favorecendo a manutenção da obesidade (ORAVA et al., 2013; SOLER-VÁZQUEZ et al., 2018; VIJGEN et al., 2012). Nesse sentido, estudos demonstraram que intensificação e / ou restauração da termogênese no TAM representa um potencial mecanismo contra a obesidade e distúrbios metabólicos associados, podendo contribuir para o aumento do gasto energético, redução da massa corporal e melhora do status metabólico (LUIJTEN et al., 2019; SAITO, 2013; SOLER-VÁZQUEZ et al., 2018). Somado a isto, evidências mostram também que o consumo exagerado de gordura induz a ativação de células não neuronais como astrócitos e microglia para produzir inflamação hipotalâmica (FRITSCHE, 2015; GUPTA et al. 2012; VALDEARCOS et al. 2017) e que o comprometimento da atividade hipotalâmica é capaz de alterar circuitos hormonais e neuronais que regulam a homeostase energética, causando obesidade e suas comorbidades (VALDEARCOS; XU; KOLIWAD, 2015).

Tendo em vista o alto número de indivíduos com sobrepeso e obesidade, há uma necessidade clara de compreender melhor os mecanismos envolvidos na gênese e fisiopatologia da obesidade. Considerando que os modelos de programação escolhidos para o presente estudo apresentam um fenótipo de obesidade, alterações hormonais, alteração no padrão alimentar e afetam funcionamento de órgãos importantes, como fígado (SOUZA;

MOURA; LISBOA, 2020; MIRANDA; MOURA; LISBOA, 2020), existe uma lacuna no que diz respeito ao TAM e inflamação hipotalâmica nesses modelos experimentais, órgãos importantes para regulação da homeostase energética, com potencial terapêutico para a prevenção da obesidade. Portanto consideramos relevante investigar o TAM, órgão endócrino ativo em adultos capaz de modular o gasto energético através da termogênese, e o hipotálamo por ser um local de regulação de gasto energético e ingestão alimentar, onde alterações nessa circuitaria se associam a distúrbios metabólicos, como resistência a leptina e insulina.

Para isso, levantamos as seguintes perguntas experimentais:

a) a exemplo do observado com a nicotina isolada, a exposição a fumaça de cigarro durante o período de amamentação é capaz de comprometer a função do hipotálamo na vida adulta, nos animais machos e fêmeas;
b) a exposição neonatal a fumaça de cigarro é capaz de influenciar a função do TAM, justificando a maior adiposidade, nos animais machos e fêmeas;
c) a exposição precoce a nicotina isolada é capaz de influenciar a função do TAM e do TAB nas proles de ambos os sexos;
d) o desmame precoce é capaz de influenciar a função do TAM, justificando a maior adiposidade nos animais machos e fêmeas quando adultos.

2 **OBJETIVO**

2.1 **Objetivo geral**

Investigar a capacidade termogênica do TAM, inflamação hipotalâmica e expressão de neuropeptídios reguladores do apetite em modelos de programação para obesidade, a saber: tabagismo materno durante a amamentação e do desmame precoce.

2.1 **Objetivos específicos**

Avaliar os efeitos da exposição exclusiva à nicotina durante a lactação sobre os descendentes machos e fêmeas aos 120 e 180 dias de vida sobre:

a) peso corporal, peso do TAM e do TAB, e leptina plasmática;
b) atividade do sistema nervoso simpático em TAM e TAB;
c) citoarquitetura do TAM e TAB, caracterizando o conteúdo de gotículas lipídicas no TAM e área de adipócitos;
d) conteúdo proteico de biomarcadores do TAM relacionados a capacidade termogênica (UCP1, receptor β3-adrenérgico, receptores de hormônios tireoidianos, PGC1α, CPT1a, AMPK e pAMPK);
e) conteúdo proteico de biomarcadores do TAB relacionados a lipogênese (ACC e FAS) e adipogênese (PPARγ, CEBPβ).

Avaliar os efeitos da exposição direta e indireta da prole à fumaça de cigarro durante a lactação sobre os descendentes machos e fêmeas aos 180 dias de vida sobre:

a) peso e gordura corporal, peso do TAM, ingestão alimentar e hormônios sexuais;
b) atividade do sistema nervoso simpático no TAM;
c) citoarquitetura do TAM, caracterizando o conteúdo de gotículas lipídicas;

d) conteúdo proteico de biomarcadores do TAM relacionados a capacidade termogênica (UCP1, receptor β3-adrenérgico, receptores de hormônios tireoidianos, PGC1α e CPT1a).
e) expressão de reguladores hipotalâmicos da termogênese no TAM (AMPK, pAMPK, POMC e MC4R);
f) via da leptina no hipotálamo total;
g) expressão de neuropeptídios hipotalâmicos reguladores da homeostase energética;
h) inflamação hipotalâmica, através de marcadores de astrócitos e microglia.

Avaliar os efeitos do desmame precoce farmacológico e não farmacológico sobre os descendentes machos e fêmeas aos 180 dias de vida sobre:

a) peso e gordura corporal e peso do TAM.
b) atividade do sistema nervoso simpático no TAM.
c) citoarquitetura do TAM, caracterizando o conteúdo de gotículas lipídicas.
d) conteúdo proteico de biomarcadores do TAM relacionados a capacidade termogênica (UCP1, receptor β3-adrenérgico, receptores de hormônios tireoidianos, PGC1α, CPT1a, AMPK e pAMPK).
e) expressão de reguladores hipotalâmicos da termogênese no TAM (AMPK, pAMPK e MC4R).

3 **MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO**

Os subitens “materiais e métodos”, “resultados” e “discussão” serão apresentados sob a forma de quatro (4) artigos originais já publicados, conforme informados a seguir:

3.1 **Artigo 1: Neonatal Tobacco Smoke Reduces Thermogenesis Capacity in Brown Adipose Tissue in Adult Rats (artigo publicado)**

T C Peixoto, E G Moura , E Oliveira, V Younes-Rapozo, P N Soares , V S T Rodrigues, T R Santos, N Peixoto-Silva, J C Carvalho, C Calvino, E P S Conceição, D S Guarda, S Claudio-Neto, A C Manhães, P C Lisboa

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Neonatal tobacco smoke reduces thermogenesis capacity in brown adipose tissue in adult rats

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Abstract

Maternal smoking is a risk factor for progeny obesity. We have previously shown, in a rat model of neonatal tobacco smoke exposure, a mild increase in food intake and a considerable increase in visceral adiposity in the adult offspring. Males also had secondary hyperthyroidism, while females had only higher T4. Since brown adipose tissue (BAT) hypofunction is related to obesity, here we tested the hypothesis that higher levels of thyroid hormones are not functional in BAT, suggesting a lower metabolic rate. We evaluated autonomic nerve activity in BAT and its function in adult rats that were exposed to tobacco smoke during lactation. At birth, litters were adjusted to 3 male and 3 female pups/litter. From postnatal day (PND) 3 to 21, Wistar lactating rats and their pups were divided into SE group, smoke-exposed in a cigarette smoking machine (4 times/day) and C group, exposed to filtered air. Offspring were sacrificed at PND180. Adult SE rats of both genders had lower interscapular BAT autonomic nervous system activity, with higher BAT mass but no change in morphology. BAT UCP1 and CPT1a protein levels were decreased in the SE groups of both genders. Male SE rats had lower β3-AR, TRα1, and TRβ1 expression while females showed lower PGC1α expression. BAT Dio2 mRNA and hypothalamic POMC and MC4R levels were similar between groups. Hypothalamic pAMPK level was higher in SE males and lower in SE females. Thus, neonatal cigarette smoke exposure induces lower BAT thermogenic capacity, which can be obesogenic at adulthood.

Key words: Tobacco smoke; Suckling period; Developmental plasticity, Obesity; Autonomic function

Introduction

The “developmental origins of health and disease” (DOHaD) concept relates the influence of disturbances during a critical window of development on the induction of permanent changes that determine a pattern of health or disease later in life (1). Evidence indicates that environmental and nutritional changes during periods of great plasticity (intrauterine life and/or lactation) induce metabolic disorders, for instance obesity, in the offspring. This phenomenon is known as metabolic programming or developmental plasticity (2,3).

Exposure to cigarette smoke during gestation and lactation can lead to developmental plasticity (4). Epidemiological data showed that maternal smoking is a risk factor for obesity in the progeny (5). Nicotine, the main psychoactive compound of the cigarette smoke, acts as an “endocrine disruptor” during suckling, promoting epigenetic changes that result in altered metabolic patterns at adulthood (6). Our research group has been studying the effect of tobacco smoke exposure exclusively during lactation and we have demonstrated that this insult can cause obesity development and endocrine disorders in the adult offspring (7,8). In this model, adult offspring have higher total body fat and visceral fat despite a mild hyperphagia, suggesting that they are hypometabolic. Surprisingly, males show secondary hyperthyroidism (higher TSH, T4, and T3) and increased adrenal catecholamines content (7), which apparently suggest a hypermetabolic status, whereas females show only higher serum T4 without presenting changes regarding catecholamines (9). All these changes are capable of altering the brown adipose tissue (BAT) thermogenesis (10).

BAT hypofunction is related to obesity since it regulates body temperature and energy expenditure. This type of adipocyte has a large number of mitochondria rich in uncoupling protein 1 (UCP1) (11). UCP1 is capable of decoupling the mechanism of oxidative phosphorylation from the respiratory chain, producing heat and thereby increasing thermogenesis (12). Non-shivering thermogenesis

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is regulated mainly by sympathetic nervous system and thyroid hormones (TH). Thus, beta 3-adrenergic receptor (β3-AR) (13) and the TH receptors TRβ1 and TRα1 (14) are considered BAT biomarkers. Type 2 iodothyronine deiodinase (Dio2) is responsible for local T4 to T3 conversion and is regulated by adrenergic stimulation (15). Other mediators act increasing or decreasing thermogenesis, such as the peroxisome proliferator-activated receptor-coactivator (PGC1α) that regulates the mitochondrial biogenesis and respiratory function (16), and the carnitine palmitoyltransferase 1A (CPT1a), which controls the fatty acid oxidation (17). Recently, Fan et al. showed, in a model of maternal prenatal and lactation nicotine exposure, morphological changes in the BAT, such as lower number of mitochondria and a whitening phenotype associated with lower expression of PGC-1α and UCP1, characterizing BAT hypofunction in the adult male rat offspring (18).

Energy balance also involves a complex network of central nervous system communication with peripheral tissues. The hypothalamus participates in the control of food intake and energy expenditure, regulating BAT thermogenesis (19). An important region in this control is the ventromedial nucleus of the hypothalamus (VMH), whose actions are mediated via the AMP-activated protein kinase (AMPK), which decreases the thermogenesis (20). The AMPK-mediated thermogenesis is mainly inhibited by thyroid hormones of the VMH (21). Another central regulator of BAT thermogenesis is the melanocortin system, which has neural projections stimulating the sympathetic nervous activity of BAT, improving thermogenesis (22). The alpha-melanocyte-stimulating hormone (α-MSH) produced in the arcuate nucleus (ARC) is derived from the reparaventricular nucleus (PVN).

As mentioned, we have characterized a gender dimorphism in thyroid and adrenal function of rats programmed by neonatal tobacco smoke exposure (7–9). Although males have higher TH and females have higher T4, they may have TH resistance in BAT. We hypothesized that the exposure to cigarette smoke during lactation can induce BAT hypofunction at adulthood, promoting fat accumulation in both genders. Thus, we investigated the long-term repercussions of tobacco smoke exposure during lactation on: 1) BAT sympathetic nerve activity, 2) BAT morphology, 3) BAT biomarkers of catecholamine and TH sensitivity (UCP1, β3-AR, TRα1, TRβ1, and Dio2), mitochondrial biogenesis (PGC1α) and fatty acid oxidation (CPT1a), and 4) hypothalamic regulators of BAT thermogenesis (AMPK, POMC, and MC4R).

Material and Methods

The Animal Care and Use Committee of the Biology Institute of the Universidade Estadual do Rio de Janeiro approved our experimental design (CEUA/019/2014). In accordance with the Brazilian Law No. 11.794/2008, experiments were done to minimize the number of rats and the suffering caused by the experimental procedures, following the ethical guideline of the three "Rs" - reduction, refinement, and replacement.

Model of direct tobacco smoke exposure during lactation

Wistar rats were housed under controlled temperature (23 ± 1°C), photoperiod of 12 h (light/dark cycle), and free access to water and food. For mating, twenty adult female rats (200–225 g) were placed with ten adult male rats (300–325 g) for 5 days. After this, sixteen pregnant rats were housed in individual cages until delivery. After birth, which was considered as the first postnatal day (PND1), all litters were adjusted to 6 pups for each dam (3 females and 3 males). As depicted in Figure 1, three days after birth, lactating dams and their offspring were randomly divided into two groups: smoke-exposed (SE, n=8) and control (C, n=8).

The SE group was directly exposed throughout lactation (until PND21) to tobacco smoke in a cigarette-smoking machine (TE-10, Teague Enterprises, USA), 4 times per day (1 h each exposure), as previously reported (7,8). This machine generated tobacco smoke from type 3R4F research cigarettes (nicotine=0.73 mg/cigarette; Reference Cigarette Program, University of Kentucky, USA). The levels of the main nicotine metabolite, cotinine, have previously been measured in the dams' serum and milk as well as in the offsprings' serum in our model (7,8) and represent the cotinine levels detected in heavy smokers (23). The C group was exposed for the same period to ambient air in a chamber similar to the one used for the SE-group exposure.

Weaning occurred at PND21 and then male and female pups were kept in different boxes (3 males and 3 females) until PND180. From PND21 to PND180

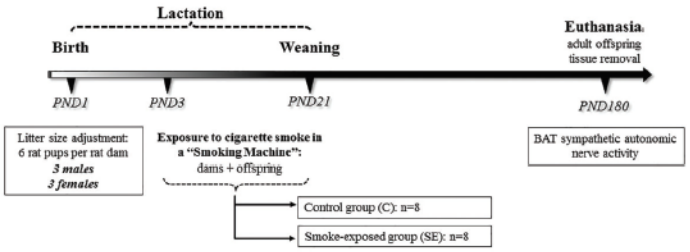

Figure 1. Experimental timeline. PND: postnatal day; BAT: brown adipose tissue.

(euthanasia day), food intake and body mass were evaluated every 4 days. The estrous cycle was analyzed after PND150. Females were euthanized during diestrous. SE and C females had regular 4- to 5-day estrous cycles, indicating that the programming by tobacco smoke exposure does not affect the reproductive cycle (9).

Autonomic nervous system (ANS) activity on interscapular brown adipose tissue (iBAT)

At PND180, one animal from each litter per group (C and SE) was fasted for 12 h and then anesthetized (ketamine 70 mg/kg and xylazine 7 mg/kg) for *in vivo* autonomic nerve electrical activity assessment as previously described (24). Interscapular BAT sympathetic nerve activity (SNA) from the left interscapular nerve was exposed under a dissection microscope. The branches were placed on a pair of hook platinum electrodes connected to an electronic device (Bio-Amplificator, Insight®, Brazil) to record the electrical signals. In order to avoid dehydration, the nerve was covered with mineral oil. Nerve activity was amplified (10,000×) and filtered (cut-off: 60 kHz). Results were analyzed using the PowerLab data acquisition system (8SP; AD Instruments, Australia). All nerve activity recordings were carried out inside a Faraday cage to avoid electromagnetic interference. Animals were kept under a warming light. After 10 min of stabilization, the average number of spikes per 10 s intervals during a 10-min period was calculated (24). The background noise level was determined in a nerve segment.

Euthanasia and tissue collection

After the measurement of iBAT ANS activity, rats were euthanized by cardiac puncture. The gonadal fat mass was collected and weighed, representing the visceral fat deposit. BAT was dissected, weighed, and prepared for morphological studies or molecular measurement (kept at –80°C). The whole brain was removed and stored at −80°C until dissection of the nuclei of interest.

BAT morphological analysis

BAT samples were fixed in formalin (freshly prepared in 1.27 M formaldehyde, 0.1 M phosphate-buffered saline, pH 7.2) and embedded in Paraplast Plus (Sigma-Aldrich, USA) for non-serial 5-μm thick sections. These sections were placed onto glass slides for hematoxylin/eosin staining and digital images were acquired randomly (TIFF format, 36-bit color, 1,360×1,024 pixels) using an Olympus DP71 camera and an Olympus BX40 epifluorescence microscope (Olympus, Japan). At least 10 photomicrographs per animal were randomly measured with the software Image-Pro Plus 5.0 (Media Cybernetics, USA). Photomicrographs were used for selection of fat droplets. The digital images of the droplets were analyzed and their areas calculated; the resulting data are shown in a histogram.

Isolation of the hypothalamic nuclei

We used a cryostat (Hyrax C52, Zeiss, Germany) to obtain the coronal sections of the brain. The ARC (Bregma –1.6 to –2.6 mm), PVN (Bregma –1.8 to –2.1 mm), and VMH (Bregma –1.8 to –3.2 mm) were isolated in accordance with the coordinates described in the Paxinos and Watson stereotaxic atlas (25). The samples were frozen (at –80°C) for western blotting.

Western blotting analysis

Protein content in the iBAT, PVN, and VMH was evaluated by western blotting. Briefly, BAT, PVN, and VMH were homogenized in RIPA buffer [50 mM Tris-HCl; pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF] and protease inhibitor cocktail (F. Hoffmann, La Roche Ltd., Switzerland). BAT was sonicated (three pulses of 5 s with 40% amplitude, intercalated by 15 s off). The homogenates were centrifuged three times (18,506 *g*, 4°C, 5 min) and the intermediate phase of the supernatant was collected after each centrifugation. At the end of the third centrifugation, the supernatant collected was adjusted to the final volume of 700 μL with RIPA buffer. The PVN and VMH were sonicated (two pulses of 10 s with 40% amplitude, intercalated by 15 s off). Protein concentration in the supernatants was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Then, homogenates were analyzed by SDS-PAGE using 30 mg total protein for BAT and 10 mg total protein for PVN. Both samples were transferred onto PVDF membranes (Hybond ECL; Amersham Pharmacia Biotech, UK). Membranes were incubated with Tris-buffered saline (TBS) containing 5% albumin for 45 min. Subsequently, the membranes were washed with TBS then incubated with specific primary antibodies: anti-UCP1 (1:500, Sigma-Aldrich, USA), anti-TRβ1, anti-TRα1 (1:500, Abcam, UK), anti-β3-AR (1:500, Santa Cruz Biotechnology, Inc., USA), anti-PGC1α, and CPT1a (1:1000, Santa Cruz Biotechnology, Inc.) overnight at 4°C. The same procedure was performed with the PVN and VMH membranes, which where incubated with anti-MC4R (1:500, Abcam) and anti-AMPKα or anti-phospho-AMPKα (1:500, Cell Signaling Technology, Inc., USA), respectively. The primary antibody anti-β−actin was used as internal control for each membrane (1:500, Sigma-Aldrich). Membranes were washed three times with Tween-TBS (0.1%) and then incubated for 1 h with the appropriate concentration of the secondary antibody (1:1000, 1:7000 or 1:1000) conjugated with biotin (anti-rabbit, anti-mouse or anti-goat Sigma-Aldrich) at room temperature. Then, the membranes were washed again three times with Tween-TBS (0.1%), which was followed by the incubation with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories, USA). Protein bands were visualized by chemiluminescence (Kit ECL plus, Amersham Biosciences, UK) followed by exposure to ImageQuant LAS (GE Healthcare, UK). The area and

density of the bands were quantified by Image J software (Wayne Rasband National Institute of Health, USA) and normalized against the bands obtained for β-actin. Results are reported as the relative percentage (%) of the control group (C).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Tissues were previously stored in RNAlater (Qiagen, USA) at –80°C to avoid RNA degradation. Total RNA was extracted from the iBAT, under RNAse-free conditions, using the RNeasy® Lipid Tissue Mini Kit (Qiagen) in accordance with manufacturer's recommendations. The quantity and the quality of the RNA samples were evaluated using the NanoVueTMPlus Spectrophotometer (GE Healthcare, England). Then, each RNA sample was diluted to obtain the final concentration of 1 μg/μL. Before the cDNA construction, RNA samples were treated with DNAse (RQ1 RNase-Free DNase- Promega, USA). The cDNA was constructed from the total RNA using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) for RT-PCR and Oligo (dT) 15 Primer (Promega). The mRNA expression was analyzed by real-time RT-PCR carried out in triplicate for each sample using an Applied Biosystems 7500 Real-Time PCR System (Applied BioSystems, USA). In order to ensure no amplification of genomic DNA, we performed a minus RT reaction (RT-) in all real-time PCR assays and no amplification product (Cq value) was detected in any of the RT-control reactions. The mRNA level of Dio2 (Assay ID: Rn00581867_m1) expression was evaluated using TaqMan® Fast Universal PCR 11 Master Mix (2X) AmpErase® UNG (Catalog #4324018; Applied Biosystems®, USA) according to the recommendations of the manufacturer. The co-amplification of β-actin gene (Assay ID: Rn00667869_m1) was performed as an internal control in all samples. This gene has been chosen as the reference gene since there were no statistical difference between the Cq mean of the control group and the smoke group. Assays were performed in triplicate and the results were analyzed using the ΔΔCT method.

Immunohistochemistry

We used one male and one female per litter of both groups, (n=7/group) for immunohistochemistry analysis. They were anesthetized with 70 mg/kg body weight ketamine and 7 mg/kg body weight xylazine and intracardially perfused with 0.9% saline, followed by 4% paraformaldehyde (in phosphate buffer, pH 7.4), and then by the same fixative plus 10% sucrose. After dissection, brains were immersed in phosphate buffer containing 20% sucrose overnight at 4°C for cryoprotection. Specimens were then frozen and coronally sectioned in 20 μm sections in a cryotome at –20°C (Hyrax C52, Zeiss) in Tissue-Tek O.C.T. compound (Sakura). Sections containing the hypothalamus, specifically the ARC region (Bregma –1.6 to –2.6 mm), according to Paxinos and Watson (25), were collected in gelatinized slides and stored at –20°C. To perform the immunohistochemistry, slides were treated with 0.3% PBS-Triton X-100 followed by a blocking solution (5% bovine serum albumin from Sigma) for 1 h at room temperature. We proceeded with incubation with anti-POMC antibody (from Santa Cruz, produced in rabbit, diluted in 1% bovine serum albumin, 1:100) overnight at 4°C. Immunoreaction was visualized by the secondary antibody anti-rabbit conjugated with ALEXA FLUOR 488 (produced in donkey, diluted 1:400; Invitrogen, USA) for 1 h at room temperature. After rinses with PBS, slides were mounted in ProLong Gold antifading reagent with 40,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Molecular Probes, USA). Omission of the primary antibodies with inclusion of the secondary antibody was also performed for the negative control procedure. Images were captured using an epifluorescence microscope (BX-40; Olympus, Japan). For the quantification of POMC, positive cells were counted in the captured images for both male and female analysis (four slices counterstained with DAPI per nucleus per animal).

Statistical analysis

Data were analyzed with the statistical program GraphPad Prism 5.0 for Windows (GraphPad Software, USA) and reported as means ± SE. First, each variable was analyzed by two-way ANOVA, with group (C *vs* SE) and gender (males *vs* females) as between-subject factors. If the initial analysis indicated both group and gender effects or interaction between these factors, then data were re-examined by one-way ANOVA followed by Newman-Keuls post-hoc test. On the other hand, if the initial analysis indicated only a group effect, data were analyzed using Student's unpaired *t*-tests, evaluating separately the developmental plasticity effects in males and females. Differences were considered significant at $P<0.05$ (Table 1).

Results

Biometric parameters

As shown in Table 2, both genders of the SE group presented higher food intake during development compared to the respective controls (males: +7%; females: +6%, $P<0.05$), but only SE females had increased body mass at PND180 (+9% *vs* C, $P<0.05$). Regarding fat deposit, a group effect was observed ($F_{1,36}=8.76$). The SE group showed higher gonadal fat mass (+41% *vs* C males; +55% *vs* C females, $P<0.05$). The iBAT mass also showed group ($F_{1,34}=12.18$) and gender ($F_{1,34}=13.33$) effects. The SE group had higher iBAT mass (+51% *vs* C males, +31% *vs* C females, $P<0.05$; +53% *vs* SE females, $P<0.05$).

BAT SNA

An interaction was observed between group and gender ($F_{1,20}=4.46$). Both genders showed group effects

Discussion

In the present study, we have demonstrated that exposure to cigarette smoke exclusively during lactation was capable of causing BAT hypofunction in the adult offspring of both genders, reducing autonomic nerve activity and important markers related to thermogenesis. Since SE males had higher serum thyroid hormones (TH) (7–9), they are probably not functional in BAT. Thus, we suggest iBAT function, causing whitening, reduction of functional mitochondria, and reduction in UCP1, PGC1α, CPT1a, and in other thermogenic-related markers. Here, male and female SE animals also showed reduced thermogenic biomarkers levels. Thus, although tobacco smoke contains thousands of substances, it is possible that nicotine plays the most important role in this programming effect. Fan et al. (18) found marked morphological changes that are possibly explained by the wider window of exposure,

Table 1. Long-term effects of neonatal cigarette smoke exposure on all analyzed parameters in 180 postnatal day offspring.

Parameters	Group effect	Gender effect	Interaction
Gonadal fat mass (g)	Yes (F = 8.76, P = 0.005)	No (F = 2.61, P = 0.114)	No (F = 0.57, P = 0.454)
iBAT SNA	Yes (F = 10.14, P = 0.004)	Yes (F = 11.91, P = 0.002)	Yes (F = 4.46, P = 0.047)
Lipid droplets (% area)	No (F = 0, P = 0.987)	Yes (F = 9.9, P = 0.005)	No (F = 0.14, P = 0.716)
UCP1 content	Yes (F = 12.47, P = 0.001)	No (F = 0.52, P = 0.478)	No (F = 0.14, P = 0.478)
β3-AR content	Yes (F = 5.99, P = 0.022)	No (F = 0.75, P = 0.397)	No (F = 0.75, P = 0.397)
TRα1 content	Yes (F = 4.91, P = 0.039)	No (F = 0.80, P = 0.381)	No (F = 0.54, P = 0.470)
TRβ1 content	Yes (F = 5.19, P = 0.034)	No (F = 1.36, P = 0.258)	No (F = 0.06, P = 0.816)
PGC1α content	Yes (F = 7.1, P = 0.013)	No (F = 0.35, P = 0.558)	No (F = 0.35, P = 0.558)
CPT1a content	Yes (F = 15.49, P = 0.0006)	No (F = 1.78, P = 0.194)	No (F = 1.78, P = 0.194)
Dio2 mRNA expression	No (F = 0, P = 0.980)	No (F = 0, P = 0.961)	No (F = 0.26, P = 0.615)
POMC (cell count)	Yes (F = 0, P = 0.984)	Yes (F = 37.12, P < 0.0001)	No (F = 1.15, P = 0.317)
MC4R level	No (F = 0,61, P = 0.442)	No (F = 1.05, P = 0.316)	No (F = 1.05, P = 0.316)
AMPK content	No (F = 0.03, P = 0.863)	Yes (F = 8.22, P = 0.007)	Yes (F = 8.22, P = 0.007)
pAMPK content	Yes (F = 4.63, P = 0.041)	Yes (F = 14.97, P = 0.0007)	Yes (F = 14.97, P = 0.0007)

Total food intake represents the sum of the total food intake between postnatal day 21 and 180. Data are reported as means ± SE, n=8/group. Comparisons were done with the control group using ANOVA. iBAT: interscapular brown adipose tissue; SNA: sympathetic nerve activity; UCP1: uncoupling protein 1; β3-AR: beta 3-adrenergic receptor; TRα1 and TRβ1: TH receptors; PGC1α: peroxisome proliferator-activated receptor-coactivator; CPT1a: carnitine palmitoyltransferase; Dio2: type 2 iodothyronine deiodinase; POMC: proopiomelanocortin; MC4R: melanocortin 4 receptor; AMPK: AMP-activated protein kinase; pAMPK: phosphorylated AMPK.

Table 2. Long-term effects of neonatal cigarette smoke exposure on body mass, food intake, visceral fat mass, and iBAT mass in 180 postnatal day-offspring.

	Males		Females	
	C	SE	C	SE
Body mass (g)	451 ± 14	462 ± 13	249 ± 7	272 ± 8*
Total food intake (kg)	3.37 ± 0.08	3.60 ± 0.05*	2.34 ± 0.03	2.48 ± 0.20*
Gonadal fat mass (g)	4.7 ± 0.4	6.7 ± 0.7*	5.5 ± 0.4	8.5 ± 0.9*
iBAT mass (g)	0.24 ± 0.03	0.36 ± 0.03*	0.18 ± 0.01	0.23 ± 0.01*

C: control; SE: smoke-exposed; iBAT: interscapular brown adipose tissue. P < 0.05, *t*-test.

($F_{1,20}$=10.14, Figure 2), as the SE group showed lower SNA at the basal condition in BAT compared to its respective controls (–58% *vs* C males; –30% *vs* C females, P < 0.05). The gender effect ($F_{1,20}$=11.91, Figure 2) is explained by the lower BAT SNA in the controls (–61% in C females *vs* C males, P < 0.05).

BAT morphology and function

As depicted in Figure 3, there was a gender effect regarding lipid droplet sectional area variable ($F_{1,21}$=9.90): SE females had smaller areas compared with SE males (–23%, P < 0.05). No group effect was observed.

Thermogenesis biomarkers showed neither interaction nor gender effects. Thus, only the group effect was considered for the variables shown below.

At PND180, both genders of the SE group showed lower UCP1 protein content (–49% *vs* C males; –74% *vs* C females, P < 0.05) and CPT1a (–45% *vs* C males; –92% *vs* C females, P < 0.05). Besides, male SE rats displayed lower protein content of β3-AR (–60% *vs* C), TRα1 (–42% *vs* C), and TRβ1 (–50% *vs* C) (P < 0.05) while female SE rats showed lower protein PGC1α expression (–67% *vs* C females, P < 0.05). The protein content of PGC1α was not altered in SE males, whereas in the SE females, β3-AR, TRα1, and TRβ1 protein contents were unchanged (Figure 4).

As shown in Figure 5, both genders in the SE group had no change of Dio2 mRNA levels at adulthood. No effects or interaction were observed regarding Dio2 gene expression.

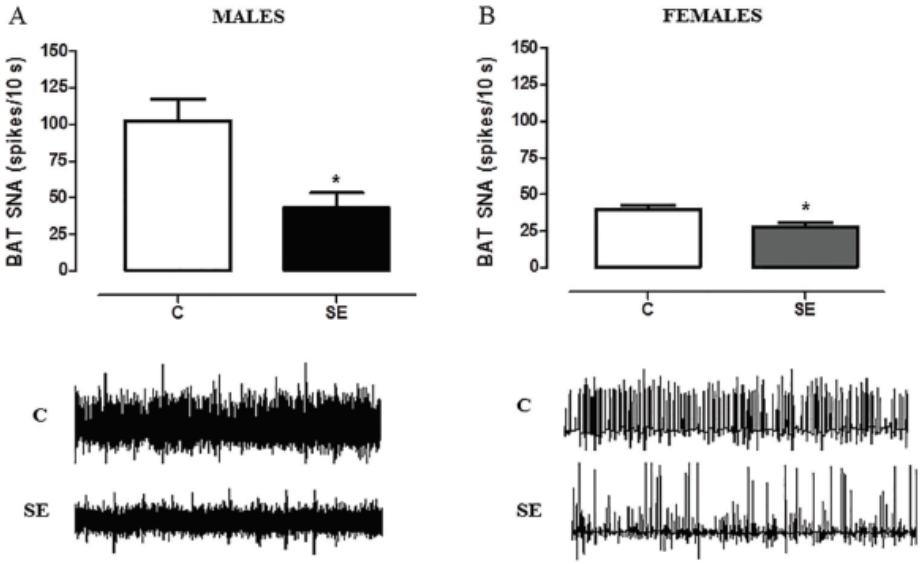

Figure 2. Long-term effects of neonatal cigarette smoke exposure on brown adipose tissue (BAT) sympathetic nerve activity (SNA). Number of spikes in 10 s at 180 postnatal days in males (*A*) and females (*B*). Representative recordings are shown at the bottom of the figures. C: control group; SE: smoke-exposed group. Data are reported as means ± SE for n=6. *P < 0.05 (two-way ANOVA re-examined by one-way ANOVA followed by Newman-Keuls post-hoc test).

Hypothalamic POMC, MC4R, and AMPK

At PND180, the POMC immunostaining in the ARC (Figure 6A–C) showed a gender effect ($F_{1,21}$=37.12) in the controls (+100% in C females *vs* C males, P < 0.05) as well as in the SE animals (+103% in SE females *vs* SE males, P < 0.05).

The MC4R protein content in the PVN (Figure 6D and E) was similar between groups and genders. There was no effect or interaction regarding this variable.

As for AMPK and pAMPK (depicted in Figure 7), interactions between factors (AMPK: $F_{1,29}$=8.22 and pAMPK: $F_{1,24}$=14.97) and gender effect (AMPK: $F_{1,29}$=8.22 and pAMPK: $F_{1,24}$=14.97) were observed. A group effect (pAMPK: $F_{1,24}$=4.63) in protein content in the VMH was also identified. SE males showed increased pAMPK content (+104%, Figure 7C, P < 0.05), whereas the SE females had decreased AMPK and pAMPK protein content in the VMH (–36 and –32%, Figure 7B and D, P < 0.05).

Discussion

In the present study, we have demonstrated that exposure to cigarette smoke exclusively during lactation was capable of causing BAT hypofunction in the adult offspring of both genders, reducing autonomic nerve activity and important markers related to thermogenesis. Since SE males had higher serum thyroid hormones (TH) (7–9), they are probably not functional in BAT. Thus, we suggest that neonatal tobacco smoke exposure programs males for TH resistance in BAT.

Although gender-related differences were not the primary focus of our evaluation, we found gender effects regarding BAT ANS only in control animals; however, no gender effect was observed on BAT biomarkers analyzed in both C and SE groups. In the literature, studies on thermogenesis and gender differences are scarce and still controversial (26–28). It has already been reported that estrogen can reduce BAT thermogenesis (26), which can explain the decrease in BAT ANS in control females compared to males.

Besides the obesity in both genders, which is in agreement with previous data (7–9), and the lower ANS in the basal condition demonstrated in the current study, postnatal cigarette smoke exposure led to an increase in iBAT weight in adult life without changing the percentage lipid droplet area in the brown adipocytes. Recently, Fan et al. (18) have demonstrated that nicotine exposure during gestation and lactation is also capable of altering iBAT function, causing whitening, reduction of functional mitochondria, and reduction in UCP1, PGC1α, CPT1a, and in other thermogenic-related markers. Here, male and female SE animals also showed reduced thermogenic biomarkers levels. Thus, although tobacco smoke contains thousands of substances, it is possible that nicotine plays the most important role in this programming effect. Fan et al. (18) found marked morphological changes that are possibly explained by the wider window of exposure,

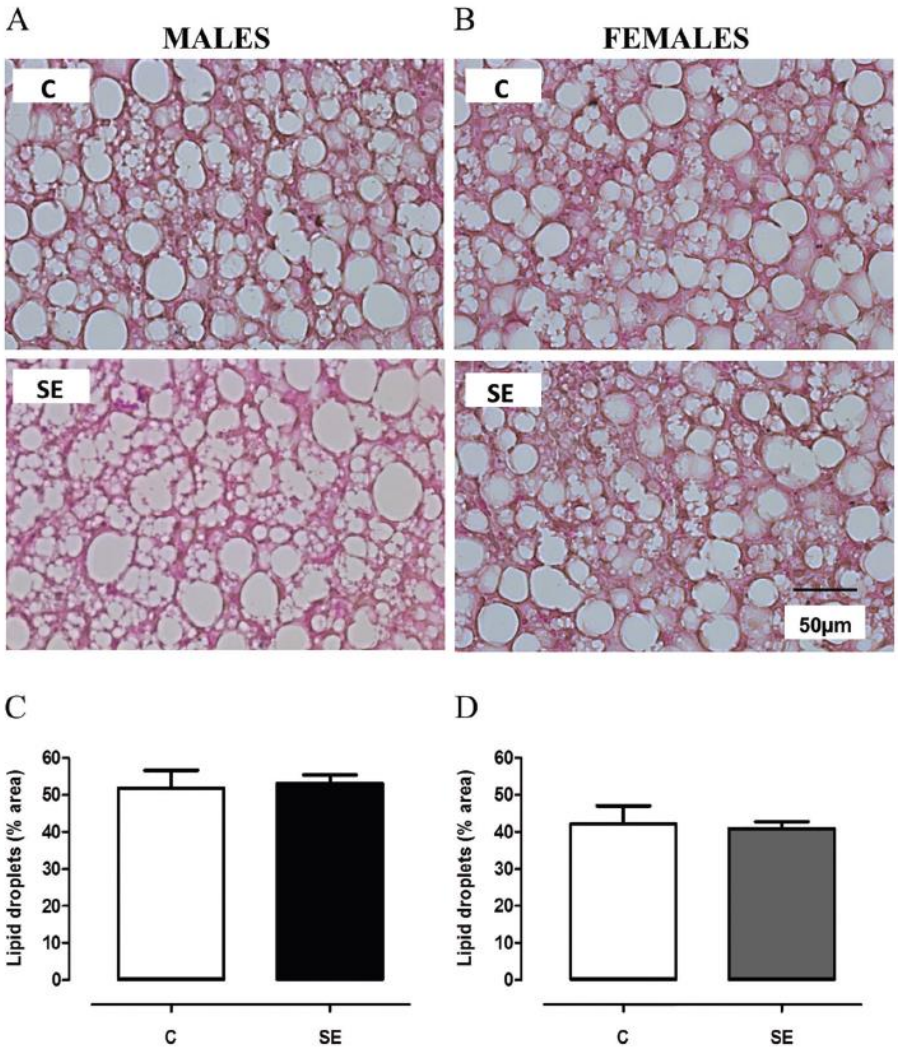

Figure 3. Long-term effects of neonatal cigarette smoke exposure on brown adipose tissue (BAT) morphology. Representative hematoxylin and eosin staining of BAT at 180 postnatal days in males (*A*) and females (*B*) (Scale bar: 50 µm). Quantitative analysis of the sectional areas of BAT lipid vacuoles is shown in males (*C*) and females (*D*). C: control group; SE: smoke-exposed group. Data are reported as means ± SE for n=6. There was gender effect, but there was no group effect for the lipid droplet sectional area (two-way ANOVA).

which encompassed both gestation and lactation. In the present study, most of the BAT adipogenesis was not influenced by smoke exposure, which might be explained by the fact that exposure in our study occurred only during lactation. It is possible that hypofunction precedes BAT whitening since another study has reported alterations in BAT function without morphological changes (29). The long-term effect of neonatal exposure to cigarette smoke on UCP1 is closely related to the reduction in thermogenic capacity and body fat gain in the SE group because UCP1 is a key molecule for the facultative thermogenesis (11,12).

The β3-AR in rodents is important for regulating BAT thermogenesis (30); therefore, the lower β3-AR content in the SE males contributes to reduce the sympathetic activity in these animals. However, no difference was observed in β3-AR content in the SE females. Despite this, the catecholamine signaling pathway can be disrupted in females.

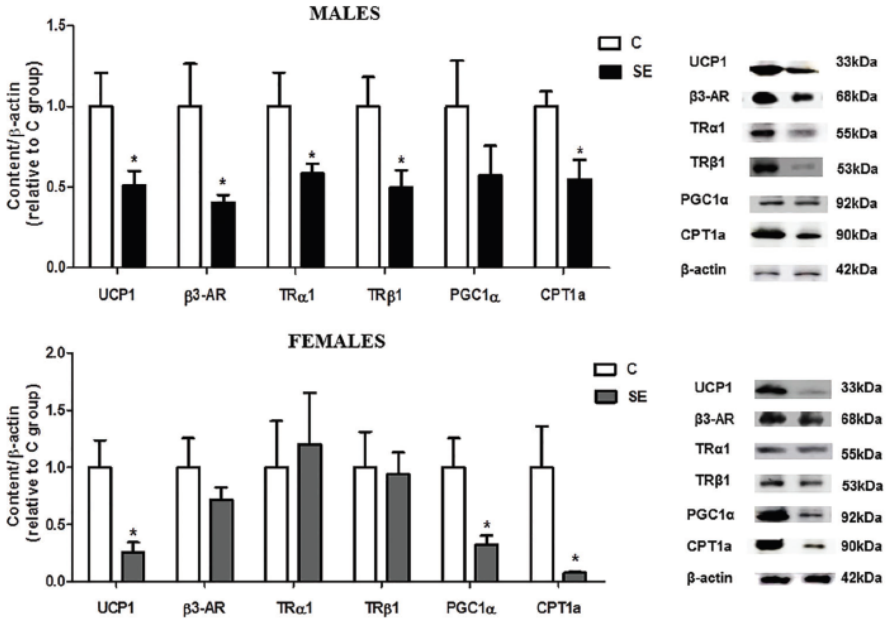

Figure 4. Long-term effects of neonatal cigarette smoke exposure on brown adipose tissue (BAT) functional parameters in male and female offspring. UCP1, β3-AR, TRα1, TRβ1, PGC1α, and CPT1a protein contents in BAT at 180 postnatal days. Representative blots of the proteins are shown beside the graphs. β-Actin content was used as control loading. C: control group; SE: smoke-exposed group. Data are reported as means ± SE for n=7–8. *P < 0.05 (*t*-test).

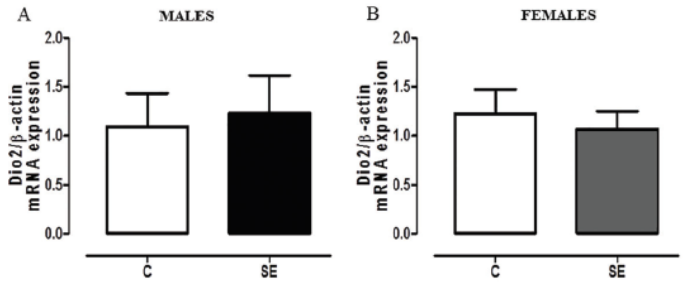

Figure 5. Long-term effects of neonatal cigarette smoke exposure on brown adipose tissue (BAT) Dio2 mRNA expression at 180 postnatal days in males (*A*) and females (*B*). C: control group; SE: smoke-exposed group. Data are reported as means ± SE for n=6–8 (*t*-test).

Another mediator of BAT thermogenesis is CPT1, a limiting enzyme in the transport of free fatty acids into the mitochondria for the oxidation and heat generation processes (31). The lower content of this enzyme in the SE groups of both genders contributes to BAT malfunctioning and increased body fat in these animals since it is known that higher CPT1a activity is related to the increase in BAT thermogenesis, increasing its oxidation rate of fatty acids (31).

Other regulators of adrenergic signaling in BAT are the thyroid hormones (TH) (32). As previously reported, male SE animals show an increase in TSH and TH levels indicating central hyperthyroidism (7). Female SE had only increased serum T4 (9). Here, we demonstrated that SE males had lower TRβ1 and TRα1, indicating TH resistance in BAT, thus contributing to a lower thermogenic action and UCP1 expression. TRβ1 isoform stimulates the expression of UCP1 and TRα1 isoform increases the adrenergic activity in BAT (14). Thus, it seems that, at least in males, TH resistance compromises autonomic nerve activity in BAT.

In our study, BAT Dio2 mRNA expression was not altered in the SE group of both genders, despite the reduction of TH receptors in the SE males. Dio2 activity/expression is responsible for T4 to T3 conversion, ensuring the cytoplasmic T3 pool (15). It is well known that plasma

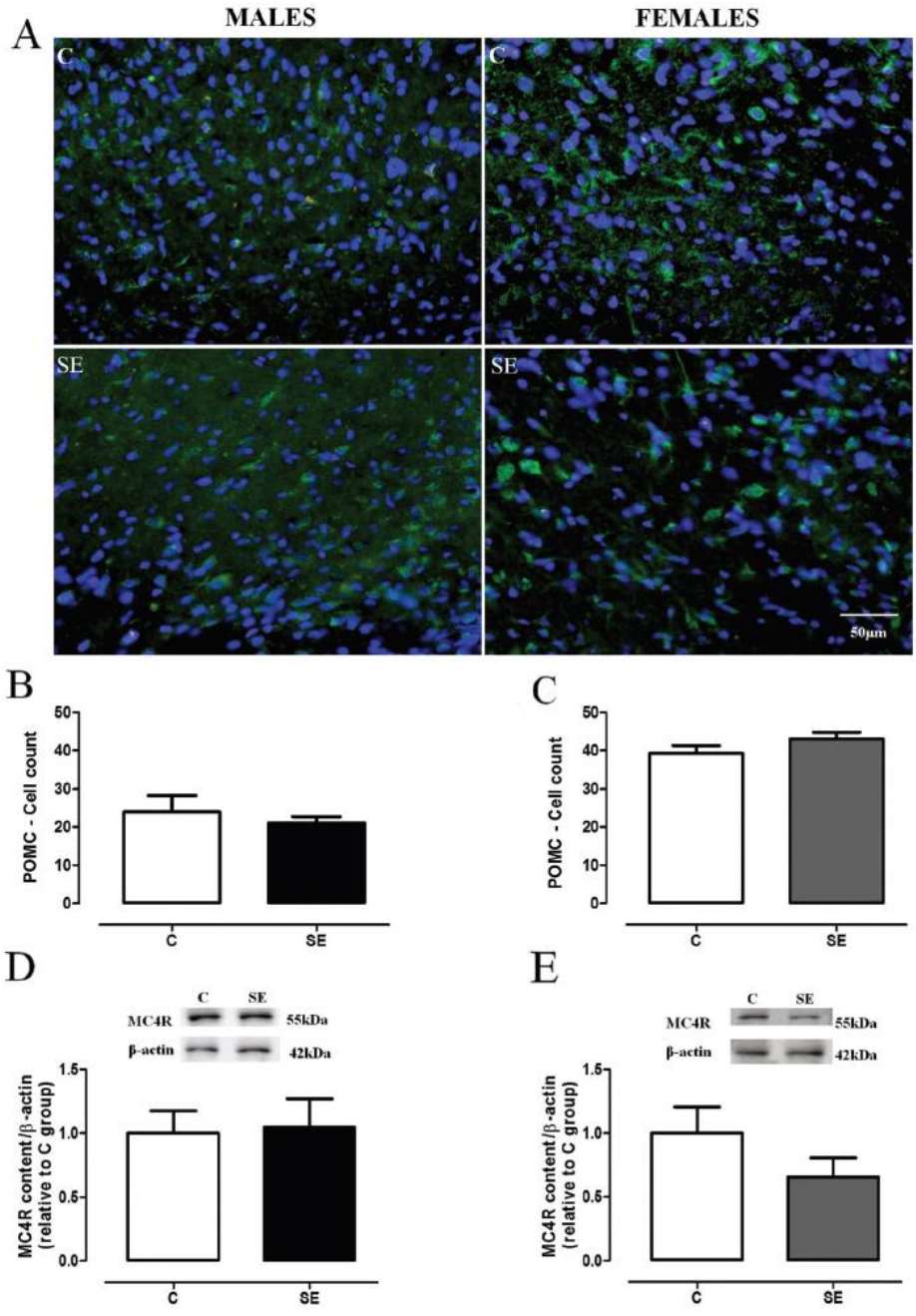

Figure 6. Long-term effects of neonatal cigarette smoke exposure on POMC and MC4R in the hypothalamus. Qualitative data of POMC (in green), counterstained for DAPI (in blue) in arcuate nucleus by immunohistochemistry (*A*) (Scale bar: 50 µm) and quantitative data concerning the number of POMC-positive cells in males (*B*) and females (*C*). Protein content of MC4R in the paraventricular nucleus (PVN) at 180 postnatal days in males (*D*) and females (*E*) with representative blots of proteins. β-Actin content was used as loading control. Data are reported as relative % to the control group. C: control group; SE: smoke-exposed group. Data are reported as means ± SE for n=7 (two-way ANOVA re-examined by one-way ANOVA followed by Newman-Keuls post-hoc test).

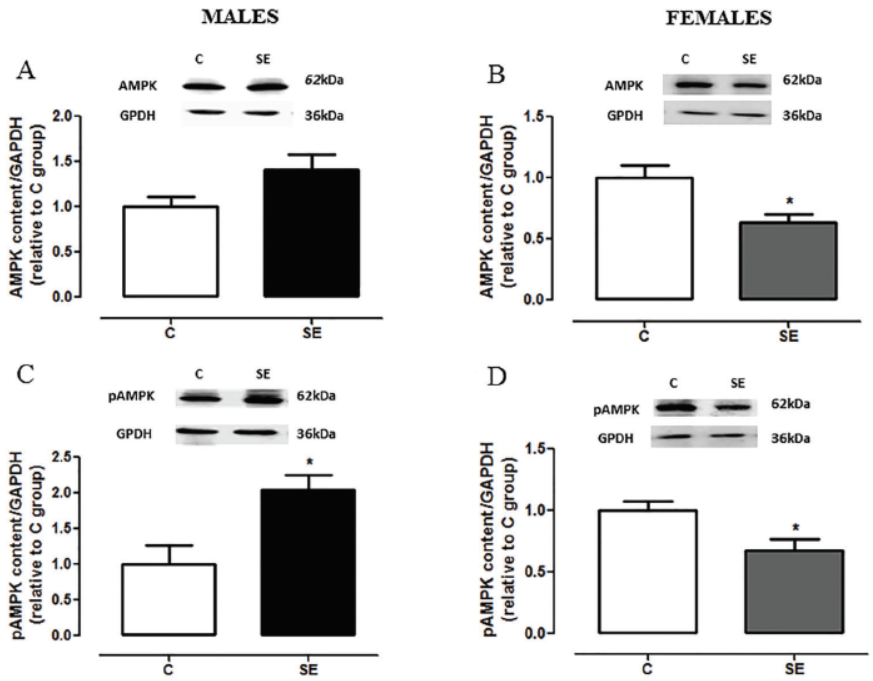

Figure 7. Long-term effects of neonatal cigarette smoke exposure on AMPK and pAMPK in the hypothalamus. AMPK (*A* and *B*) and pAMPK (*C* and *D*) protein contents in the ventromedial nucleus of the hypothalamus at 180 postnatal days in males and females. Representative blots of proteins are shown above the graphs. GAPDH content was used as loading control. Data are reported as relative % to the control group. C: control group; SE: smoke-exposed group. Results are reported as means ± SE for n=6–8. *P < 0.05 (*t*-test).

TH negatively regulate this enzyme (15). As the SE group had increased serum TH, a lower BAT Dio2 expression was expected. Thus, the unchanged Dio2 found in our model also suggests TH resistance at BAT level.

BAT PGC1α is stimulated by both adrenergic tonus and TH levels (32). In SE females, the reduction of BAT PGC1α can contribute to the reduction in thermogenic activity, since PGC1α is a transcriptional co-activator that mediates events related to energy metabolism, capable of interfering in the expression UCP1 in the BAT (33), besides acting in the control of mitochondrial biogenesis (34). Since SE females had hyperthyroxinemia, we expected to find increased BAT PGC1α. Thus, again, this is suggestive of TH resistance at least in females.

The melanocortin system activation in the PVN controls BAT ANS activity (35,36). Despite the reduction of ANS activity in SE animals, the MC4R content in the PVN as well as in the number of POMC positive cells were unaltered in our model, suggesting that the BAT ANS dysfunction was not related to melanocortin system programming.

As previously published, SE males have higher TSH and TH with increased body fat (7). Thus, the hyperthyroid status of this group was not enough to cause body mass loss. It is known that T3 inhibits AMPK in the VMH and increases sympathetic nervous system activity (21). Thus, the increase in AMPK in the VMH of SE males is suggestive of TH resistance. Also, higher AMPK content explains hyperphagia, lower SNA, and lower thermogenic markers, since AMPK enhances food intake and decreases thermogenesis (20). SE males had lower SNA associated with decreased adrenergic and TH receptors in BAT, indicating that higher TH is also not functional in this tissue. Conversely, it seems that SE females have a normal response to T4 in the hypothalamus since AMPK content was reduced. Thus, SE females have a less intense reduction of BAT SNA compared with SE males (30 *vs* 58%). Other factors may be acting on SE females that may help explain the persistent lower BAT SNA, such as leptin resistance, as suggested in a previous study (9).

Figure 8 summarizes the main findings of the current study. Here we showed that tobacco smoke exposure during the suckling period reduced the thermogenic capacity of BAT in both genders, possibly explaining the increase in body adiposity in adulthood, independently

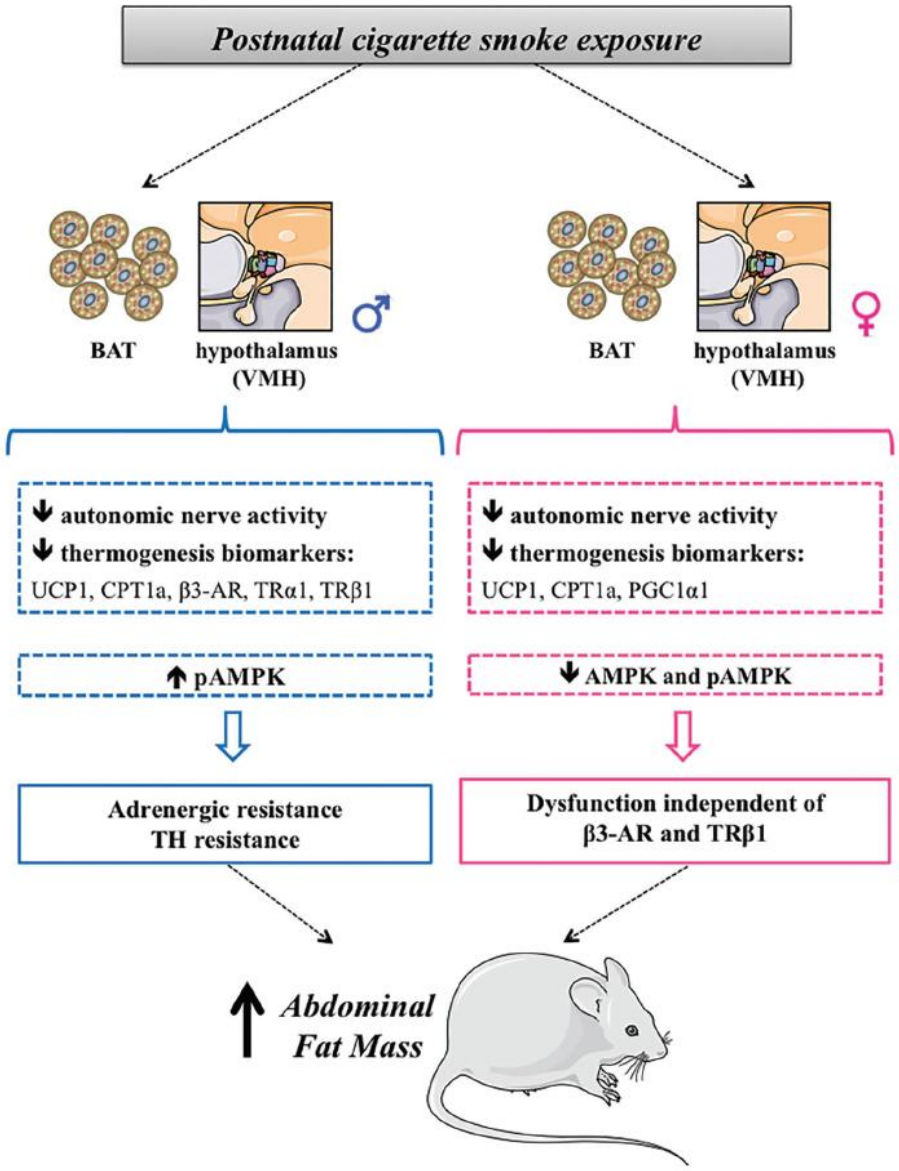

Figure 8. Smoking in critical windows of development causes epigenetic alterations and is a risk factor for adulthood obesity. BAT: brown adipose tissue; UCP1: uncoupling protein 1; β3-AR: beta 3-adrenergic receptor; TRα1 and TRβ1: thyroid hormones receptors α1 and β1; CPT1a: carnitine palmitoyltransferase 1a; PGC1α: peroxisome proliferator-activated receptor-coactivator; VMH: ventromedial nucleus of the hypothalamus; AMPK: AMP-activated protein kinase; pAMPK: phosphorylated AMPK; TH: thyroid hormone.

of BAT melanocortin system regulation. Apparently, the reduction in BAT SNA stimulation was almost twice as intense in males. In males, the main mechanism for the observed alterations can be attributed to adrenergic and TH resistance in both BAT and VMH. In females, however, this resistance is not completely clear since it seems to be present only in BAT and it is not dependent on β3-AR and TRβ1 function.

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3.2 **Artigo 2: Hypothalamic Neuropeptides Expression and Hypothalamic Inflammation in Adult Rats That Were Exposed to Tobacco Smoke During Breastfeeding: Sex-Related Differences (artigo publicado)**

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(Montgomery and Ekbom, 2002; Oken et al., 2008). Therefore, early exposure to cigarette smoke has great potential to cause epigenetic changes that lead to metabolic programming (Lisboa et al., 2012; Napierala et al., 2016).

Nicotine is the main substance responsible for maintaining cigarette addiction. Despite this, cigarette also contains thousands of different toxic substances, such as free radicals, polycyclic aromatic hydrocarbons, aromatic amines and tobacco-specific nitrosamines (Hecht, 2002). However, nicotine exposure during pregnancy and/or lactation does not always mimic all the effects of tobacco smoke programming (Oliveira et al., 2009; Oliveira et al., 2010; Chen et al., 2011; Maritz and Harding, 2011; Santos-Silva et al., 2013; Lisboa et al., 2017).

Previously, our research group demonstrated that maternal exposure to nicotine alone during lactation programs the adult offspring for obesity and hormone dysfunctions such as lower thyroid hormone and higher glucocorticoid (Oliveira et al., 2010; Pinheiro et al., 2011). Maternal nicotine also alters the hypothalamic circuitry, leading to central resistance to leptin (Oliveira et al., 2010) with increased neuropeptide Y (NPY) and corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN), increased pro-opiomelanocortin (POMC) in the arcuate nucleus (ARC), and reduced cocaine- and amphetamine-regulated transcript (CART) and thyrotropin-releasing hormone (TRH) in the PVN (Younes-Rapozo et al., 2013). In addition, nicotine was also able to alter glial cells by causing microgliosis, as indicated by higher CX3C chemokine receptor 1 (CX3CR1), and astrogliosis in the hypothalamic nuclei of the programmed adult offspring, which was characterized by higher astrocytes number and glial fibrillary acidic protein (GFAP) density (Younes-Rapozo et al., 2015a). These aforementioned findings were found in the male progeny, since females were not studied.

The hypothalamus regulates energy expenditure and food intake, so it has been a target of many studies. In hypothalamic circuitry, there are groups of neurons present in the ARC, called anorexigens, that release neuropeptides such as POMC, α-melanocyte stimulating hormone (αMSH) and CART, which are positively regulated by leptin, reducing food intake and increasing energy expenditure, and orexigenic neurons that secrete neuropeptides such as NPY and agouti-related protein (AgRP), which are negatively regulated by leptin, increasing food intake and reducing energy expenditure (Valdearcos et al., 2015). These neurons project their axons into other hypothalamic nuclei: ventromedial nucleus (VMH), paraventricular nucleus (PVN), and lateral hypothalamus (LH) (Sanchez-Lasheras et al., 2010). ARC neurons are also the main regulators of the secretion of TRH and CRH in the PVN, both anorexigenic neuropeptides, but with different action on metabolism. TRH, through thyroid hormones, is responsible for increasing thermogenesis, whereas CRH, through glucocorticoids, enhances adipogenesis (Martínez-Sánchez et al., 2014).

It has previously been shown that in the model of tobacco smoke exposure during lactation, *via* milk (indirect) and inhalation (direct), the animals of both sexes present increased body fat, hyperphagia and hyperleptinemia at adulthood (Santos-Silva et al., 2013; Lisboa et al., 2017). These three alterations are supposedly related to changes in the expression patterns of neuropeptides and hypothalamic inflammation since leptin interferes with central neuropeptides production/secretion as well as the activation of glial cells, such as astrocyte and microglia (De Souza et al., 2005; Gao et al., 2014; Valdearcos et al., 2015).

Hypothalamic astrocytes and microglia are responsible for the immune response to peripheral stimuli (Valdearcos et al., 2015). Astrocytes have an important role in maintaining the parenchymal environment in the brain and are a critical component in the regulation of the influx and efflux of several substances that are present in the brain (De Git and Adan, 2015). The constant activation of these cells involves proinflammatory signaling cascades that can alter local neural circuits and metabolic control, compromising the action of leptin and insulin, thereby impairing energy regulation (Gao et al., 2014). Astrogliosis is the inflammatory process of astrocytes and occurs in response to acute or chronic insults in the brain, such as the consumption of hyperlipidic diet and obesity, which lead to the process of hypothalamic inflammation (Abbott et al., 2006; Waise et al., 2015). Astrogliosis is a feature of numerous neuropathologies, including strokes, Alzheimer's disease, spinal cord and traumatic brain injuries (Zhang et al., 2010). It is proposed that the initial event that triggers hypothalamic leptin resistance is hypothalamic inflammation, which eventually leads to the development of obesity (Dragano et al., 2017).

Thus, considering: the amount of substances present in the cigarette smoke (1), the deleterious effects caused by the model of nicotine programming in the neuroendocrine system of adult male rats (2), the body fat accumulation associated with hyperphagia and hyperleptinemia in male and female rats in the model of tobacco smoke programming (3), that smoking affects sex hormone production and action (Kim et al., 2019), which may affect hypothalamic function and immunomodulation (4), here we decided to investigate whether early postnatal exposure to cigarette smoke is also deleterious to the neuroendocrine system of the progeny of both sexes at adulthood. The present study was designed to evaluate leptin signaling and neuropeptides expression in the hypothalamus, as well as inflammation, astrocyte and microglia markers in adult animals that were exposed to cigarette smoke only during the breastfeeding period. Also, for the first time, the circulating levels of sex hormones were determined in this experimental model.

EXPERIMENTAL PROCEDURES

Ethical approval

The Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro approved our experimental design (CEUA/019/2014). In accordance with the Brazilian Law no. 11.794/2008, experiments were done to minimize the number of rats and the suffering caused by

the experimental procedures, following the ethical doctrine of the three "Rs" — reduction, refinement and replacement.

Experimental model of tobacco smoke exposure during the breastfeeding period

Adult Wistar female rats were placed with male rats in a 2 to 1 ratio for 5 days. Animals were housed under controlled temperature (23 ± 1 °C), with a photoperiod of 12 h (light / dark cycle) and free access to water and food. After this period, pregnant rats were housed in individual cages until delivery. At birth, which was considered as the first postnatal day (PND1), all litters were adjusted to six pups per dam, three females and three males.

Three days after birth, lactating dams and their offspring were randomly divided into two groups: smoke-exposed (SE, n = 8) and control (Crtl, n = 8). Animals assigned to the SE group were passively exposed (dams and offspring together), during the lactation period (until PND21), to tobacco smoke in a cigarette-smoking machine (TE-10, Teague Enterprises, Davis, CA, USA), four times per day (1 h each exposure), as previously reported (Santos-Silva et al., 2013; Peixoto et al., 2018). This machine generates tobacco smoke from one research cigarette (3R4F) burned at a time (nicotine = 0.73 mg/cigt; Total Particulate Matter = 11.0 mg/cigt; Tar = 9.4 mg/cigt; Carbon Monoxide = 12.0 mg/cigt; Reference Cigarette Program, University of Kentucky, Lexington, KY, USA). In 1 h of smoke exposure, the machine burns five to six research cigarettes. As four 1-h periods of exposure (9:00 am, 12:00 pm, 3:00 pm and 6:00 pm) were carried out each day, we had a total of 20 to 24 cigarettes per day. Eight cages were put together at the same time in the chamber. A mixture containing 11% mainstream smoke (smoke from the puff stream) and 89% side-stream smoke (smoke released from the burning end of a cigarette) as a surrogate for active smoking was generated by the smoking machine in a staggered manner at the rate of a single 35-ml puff of 2-s duration each minute. During exposure, the total suspended particulate was measured by weighing Teflon-coated fiber filters (TX40H120-WW, Pallflex Products Co., Putnam, CT, USA) before and after a 5-min period, when air was collected from the chamber. There were 12 periods of collection, which generated levels of 24.0 ± 2.4 mg/m^3 (mean ± SEM). Dams and offspring from the Crtl group were exposed to ambient air in a similar chamber for the same period.

As aforementioned, the smoke-exposure period lasted for 19 days (from PND3–21). According to Quinn (2005), 1 day in the life of a rat is equivalent to about 9 days in human life. Thus, the period of exposure to smoke for the rat here largely covers the period of lactation in this species, and is equivalent to the exclusive breastfeeding period recommended for a child (6 months), according to World Health Organization.

Detection of cotinine (main nicotine metabolite)

The levels of serum cotinine were determined in the dams' serum and milk, as well as in the neonate offspring's serum using an ELISA kit from OraSure Technologies (Bethlehem, PA, USA) and following the manufacturer's recommendations. This measurement was performed in dams and one pup/sex/group at PND21, when the pup was killed after the first exposure in the morning on the last day in the smoke chamber.

Post-weaning development

The animals were weaned at PND21 (after the last exposure in the chamber) and female pups were separated from male ones and kept in different cages until euthanasia at PND180.The estrous cycle was analyzed after PND150. SE and Crtl females had regular 4–5-day estrous cycles. Females were killed during diestrous. Chow consumption and body mass of the offspring were recorded weekly from weaning to the day of euthanasia, at which time the amount of visceral fat was weighed to estimate central adiposity.

Immunohistochemistry analysis

The animals (n = 8 per group, one per litter/sex) were anesthetized with ketamin 70 mg/kg b.w. and xilasin 7 mg/kg b.w. and then intracardially perfused with 0.9% saline, followed by 4% paraformaldehyde (in phosphate buffer, pH 7.4) and 4% paraformaldehyde plus 10% sucrose. After dissection, brains were kept immersed overnight in phosphate buffer containing 20% sucrose, at 4 °C for cryoprotection. Then, they were frozen and coronally sectioned at 20 µm with a cryotome (Hyrax C52, Zeiss, Germany) at −20 °C in Tissue-Tek O.C.T. compound (Sakura). Sections containing the hypothalamic regions ARC, PVN and LH, according to Paxinos and Watson (1998), were collected in gelatinized slides and stored at −20 °C. To perform the immunohistochemistry, slides were treated with 0.3% PBS-Triton X-100 followed by a blocking solution (5% bovine serum albumin from Sigma) for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C. The immunoreaction was visualized by the use of a secondary antibody for 1 h at room temperature. After rinses with PBS, slides were mounted in ProLong Gold antifading reagent with 40,6-diamidino-2- phenylindole (DAPI) (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Omission of the primary antibodies with inclusion of the secondary antibody was also performed for the negative control procedure.

The specific primary antibodies that were used were: anti-NPY (1:1000 rabbit polyclonal antibody, Sigma Aldrich, Invitrogen Corporation CA, USA), anti-alpha-MSH (1:10000 sheep polyclonal antibody, EMD Millipore Corporation (Billerica, MA, USA), anti-TRH (1:400 rabbit polyclonal antibody, LifeSpan Biosciences (Seattle, Washington, USA), anti-CRH (1:100 rabbit polyclonal antibody, Abcam Inc. (Cambridge, MA, USA), anti-CART (1:100 goat polyclonal antibody, Santa Cruz, CA, USA) and anti-GFAP (1:100 mouse monoclonal antibody, Sigma Aldrich, Invitrogen Corporation CA, USA).

The primary antibodies were revealed by the appropriate secondary antibodies: donkey anti-rabbit conjugated with Alexa Fluor 488 or donkey anti-goat conjugated with Alexa Fluor 555, donkey anti-sheep conjugated with Alexa Fluor

488, all from Molecular Probes (Invitrogen, Oregon, USA), diluted 1:400.

The hypothalamic nuclei that were analyzed were: PVN, ARC and LH. The bregma levels that were used as references for the aforementioned nucleus were: PVN from −0.6 to −2.10 mm, ARC and LH from −2.10 to −3.6 mm. Images were captured using an epifluorescence microscope (BX-40; Olympus, Tokyo, Japan).

Western blotting analysis

In samples of total offspring hypothalamus, we measured the contents of proteins associated with the leptin signaling pathway (leptin receptor, OBR-b; phosphorylated Janus Kinase 2, pJAK2; phosphorylated signal transducer and activator of transcription 3, pSTAT3; suppressor of cytokine signaling 3, SOCS3), activated microglia marker (CX3C chemokine receptor 1, CX3CR1), pro-inflammatory cytokine (interleukin 6, IL6) and anti-inflammatory and antioxidant marker (α7 subunit nicotinic acetylcholine receptors, α7nAChRs). The total hypothalamus was homogenized in using RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF), which included a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), and 500 µL of the buffer was used to homogenize the tissue. The protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, CA, USA). Homogenates were analyzed by the SDS PAGE method. Samples containing 10 µg (leptin signaling and α7nAChR) or 30 µg (CX3CR1 and IL6), using a 12% polyacrylamide gels were submitted to electrophoresis and electroblotted onto a nitrocellulose membrane (Hybond P ECL membrane, Amersham Biosciences, London, UK). Membranes were incubated with Tween-TBS (0.1%) containing 5% albumin for 45 min. Subsequently, the membranes were washed with TBS and then incubated with specific primary antibodies: anti-β-actin (1:500, Sigma-Aldrich, Invitrogen, CA, USA); anti-OBR-b, anti-pJAK2, anti-pSTAT3, and anti-SOCS3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CX3CR1 (1:500, abcam plc, abcam, Cambridge, UK), anti-IL6 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α7nAChR (1:1000, Bioss Inc., Massachusetts, USA) were incubated overnight at 4 °C. Membranes were washed three times with Tween-TBS (0.1%) and then incubated for 1 h with the appropriate concentration of the secondary antibody conjugated to biotin: 1:10,000, anti-mouse (β-actin and OBR-b), anti-goat (pJAK2, pSTAT3), anti-rabbit (SOCS3) and 1:5000, anti-goat (CX3CR1) from Sigma Aldrich, anti-rabbit (α7nAChR and IL6) from Invitrogen Corporation CA, USA), at room temperature. Then, the membranes were washed again three times with Tween-TBS (0.1%), followed by incubation with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA, USA) in the same dilution as the secondary antibody. The protein bands were visualized by chemiluminescence (Kit ECL plus, Amersham Biosciences, London, UK) using the ImageQuant LAS equipment (GE Healthcare, Buckinghamshire, UK). The area and density of the bands were quantified by Image J software (Wayne Rasband National Institute of Health, MA, USA) and normalized against the bands obtained for β-actin. Results were expressed as relative (%) to the control group (Ctrl).

Radioimmunoassay (RIA) analysis

Serum sex hormones were determined by RIA commercial test kits in accordance with the manufacturer's instructions (MP Biomedicals, LLC, NY, EUA). Samples were analyzed in a single assay. The sensitivities of the assays were 0.1 ng/mL for testosterone and 10 pg/mL for estradiol. The intra-assay coefficients of variation were: 1.50 for testosterone and 1.60 for estradiol.

Statistical analysis

The data were analyzed with the statistical program GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA) and expressed as means ± standard error of the mean (SEM). Comparisons between the groups were performed using Student's unpaired t-test, because sex (males and females) was not considered as factor. Thus, only the programming effect (SE and Ctrl) was considered in the analyses. Differences were considered significant at $p < 0.05$.

RESULTS

Cotinine levels

As shown in Table 1, cotinine was detected in both milk and serum of SE dams at the end of the lactation period, as well as in the serum of SE pups of both sexes at weaning, suggesting that the regimen of smoke exposure used in our rat model approximates that of typical (moderate to heavy) human smokers (Benowitz et al., 1982; Eskenazi and Bergmann, 1995). The current cigarette exposure procedure results in cotinine concentrations in dams' serum and milk of 35 ng/ml, as well as of 41 ng/ml in the male offspring serum and of 29 ng/ml in female offspring serum's. As expected, Crtl dams and Crtl pups groups showed levels below the detection limit of the technique (<8 ng/ml).

Table 1. Cotinine levels in dams and pups at the end of breastfeeding.

DAMS		
	Crtl	SE
Milk cotinine (ng/mL)	ND	34.7 ± 6.4
Serum cotinine (ng/mL)	ND	35.0 ± 3.1
MALE PUPS		
	Crtl	SE
Serum cotinine (ng/mL)	ND	41.3 ± 7.5
FEMALE PUPS		
	Crtl	SE
Serum cotinine (ng/mL)	ND	28.8 ± 0.4

Crtl: Control; SE: Smoke-exposed. ND: Non-detectable. Cotinine levels below the detection limit of the technique (<8 ng/ml). Values represent means ± SEM, n = 8/group.

Table 2. Effect of tobacco smoke exposure during the breastfeeding period on biometric parameters and serum sex hormones at PND180.

MALES		
	Crtl	SE
Total chow intake (kg)	3.11 ± 0.05	3.40 ± 0.03
Body mass (g)	430.8 ± 10.9	444.4 ± 10.1
Visceral fat mass (g)	17.5 ± 1.4	23.9 ± 2.6
Estradiol (pg/mL)	51.9 ± 4.3	49.1 ± 4.5
Testosterone (ng/mL)	8.8 ± 1.2	7.4 ± 1.5
FEMALES		
	Crtl	SE
Total chow intake (kg)	2.15 ± 0.02	2.38 ± 0.02
Body mass (g)	240.1 ± 4.4	262.6 ± 5.7
Visceral fat mass (g)	10.9 ± 0.7	14.7 ± 0.8
Estradiol (pg/mL)	117.4 ± 9.9	91.9 ± 6.5
Testosterone (ng/mL)	0.67 ± 0.1	0.55 ± 0.1

Crtl: Control; SE: Smoke-exposed. The total chow intake represents the sum of the total food intake between PND21 and PND180. Values represent means ± SEM, n = 8/group.

$p < 0.05$ *vs* Crtl.

Biometric parameters and serum sex steroids

Table 2 depicts chow intake, body mass and adiposity, as well as circulating levels of gonadal hormones in SE offspring at PND180. As expected, SE animals from both sexes had hyperphagia (males: +9% *vs* Crtl; females: +11% *vs* Crtl, $p < 0.05$). SE females exhibited higher body mass (+9% *vs* Crtl, $p < 0.05$), while SE animals from both sexes had increased visceral fat mass (males: +36% *vs* Crtl; females: +35% *vs* Crtl, $p < 0.05$). These data are in accordance with the literature (Peixoto et al., 2018). SE males had no alteration in serum estradiol and testosterone concentrations at adulthood, while SE females had a reduction in estradiol levels (−21% *vs* Crtl, $p < 0.05$), without alteration in testosterone levels.

Leptin signaling pathway in the hypothalamus

At PND180, no significant changes were observed in the hypothalamic OBRb, pJAK, pSTAT3 and SOCS3 in the SE group of both sexes (Fig. 1A and Fig. 1B).

Hypothalamic neuropeptides

At adulthood, NPY immunohistochemistry indicated a significant increase in fiber density in the ARC of SE males (+79% *vs* Crtl, Fig. 2A and Fig. 2B, $p < 0.05$), with no change observed in either the PVN or the LH (Fig. 2C and Fig. 2D). Concerning SE females, no difference was observed in NPY immunostaining intensity (Fig. 2E, Fig. 2F and Fig. 2G).

As depicted in Fig. 3, no difference was observed in CART immunostaining intensity in the ARC, PVN and LH of SE males. SE females showed reduced number of CART-positive cells only in the ARC (−25% *vs* Crtl, Fig. 3A and Fig. 3B, $p < 0.05$), with no changes observed in the other nuclei.

SE males did not show any difference in αMSH immunostaining intensity in the hypothalamic nuclei, while SE females showed reduced immunostaining intensity for this neuropeptide in the PVN and LH (PVN: −36% *vs* Crtl and LH: −41.89% *vs* Crtl, Fig. 4A, Fig. 4B, Fig. 4 C and Fig. 4D, $p < 0.05$).

TRH immunohistochemistry indicated a reduction in fiber density in SE males in the PVN (−55% *vs* Crtl, Fig. 5A and Fig. 5B, $p < 0.05$), whereas SE females had no alteration (Fig. 5C). CRH immunohistochemistry indicated an increase in fiber density in SE males (+92% *vs* Crtl, Fig. 6A and Fig. 6B, $p < 0.05$), while SE females had no change in CRH immunostaining intensity in the PVN (Fig. 6C).

Hypothalamic inflammation

At PND180, immunohistochemistry data showed that SE males had a higher GFAP fiber density in both the ARC and PVN (ARC: +2.3 fold

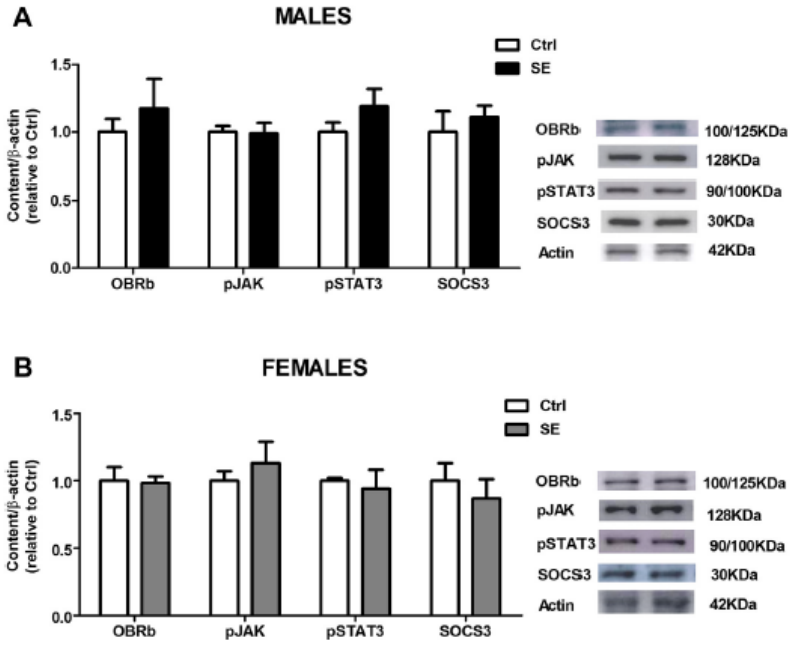

Fig. 1. Leptin signaling pathway in the hypothalamus of PND180 animals that were directly and indirectly exposed to cigarette smoke during the breastfeeding period. OBRb, pJAK, pSTAT3 and SOCS3 protein contents in the hypothalamus at PN180 in males (A) and females (B). Representative blots of the proteins are shown beside the graphs. β-Actin content was used as control loading. Values are expressed as relative (%) to the control group. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *$p < 0.05$ *vs* Crtl.

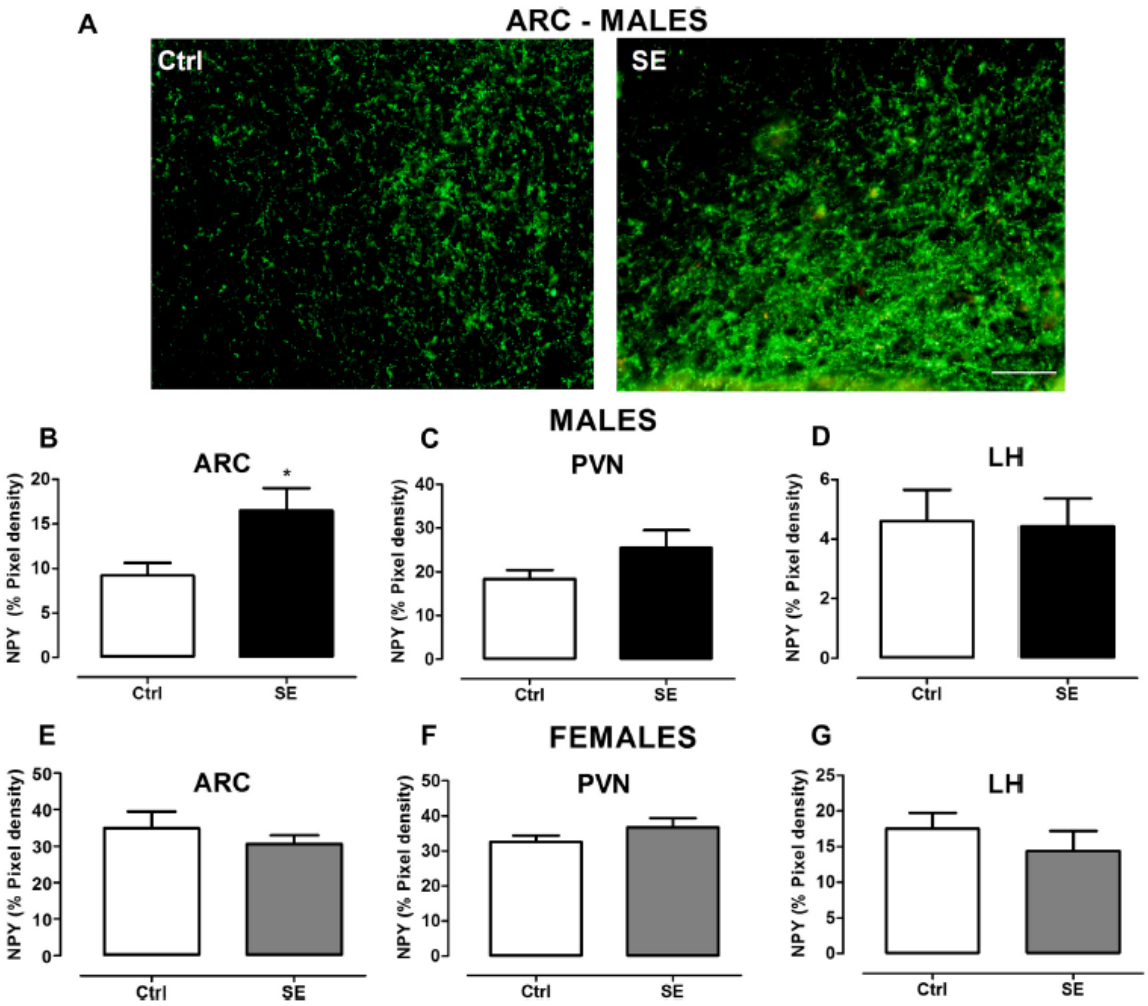

Fig. 2. Immunohistochemistry of neuropeptide Y (NPY) in the arcuate nucleus (ARC), paraventricular nucleus (PVN) and lateral hypothalamus (LH) at PND180. Immunoreaction of NPY (green) in the ARC of males (A). Fiber density quantification in the ARC (B), PVN (C) and LH (D) of males. Fiber density quantification in the ARC (e), PVN (F) and LH (G) of females. Calibration bar: 50 μm. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *p < 0.05 *vs* Crtl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vs Crtl and PVN: + 1.5 fold *vs* Crtl, Fig. 7A, Fig. 7B and Fig. 7C, $p < 0.05$), without change in the LH. No difference was observed in SE females regarding this parameter (Fig. 7D, Fig. 7F and Fig. 7E).

As depicted in Fig. 8C and Fig. 8D, SE males had a higher IL6 protein content in the total hypothalamus, whereas SE females showed no alteration of this pro-inflammatory cytokine. Regarding the protein content of CX3R1 (marker of activated microglia) and α7nAChR (anti-inflammatory marker), no change was observed in the SE animals of both sexes (Fig. 8A, Fig. 8B, Fig. 8E and Fig. 8F).

DISCUSSION

In previous studies, we demonstrated that adult animals of both sexes that were directly and indirectly exposed to cigarette smoke during breastfeeding showed visceral fat accumulation, hyperphagia and increased circulating leptin concentration (Peixoto et al., 2018). As an exclusive characteristic of male offspring, we detected hyperthyroidism and hypocorticosteronemia, while increased plasma T4 and corticosterone levels were found in females (Santos-Silva et al., 2013; Lisboa et al., 2017). Currently, in this same model, we observed that the neonatal exposure to cigarette smoke is capable of altering the hypothalamic circuitry with respect to the neuropeptides regulating food intake and

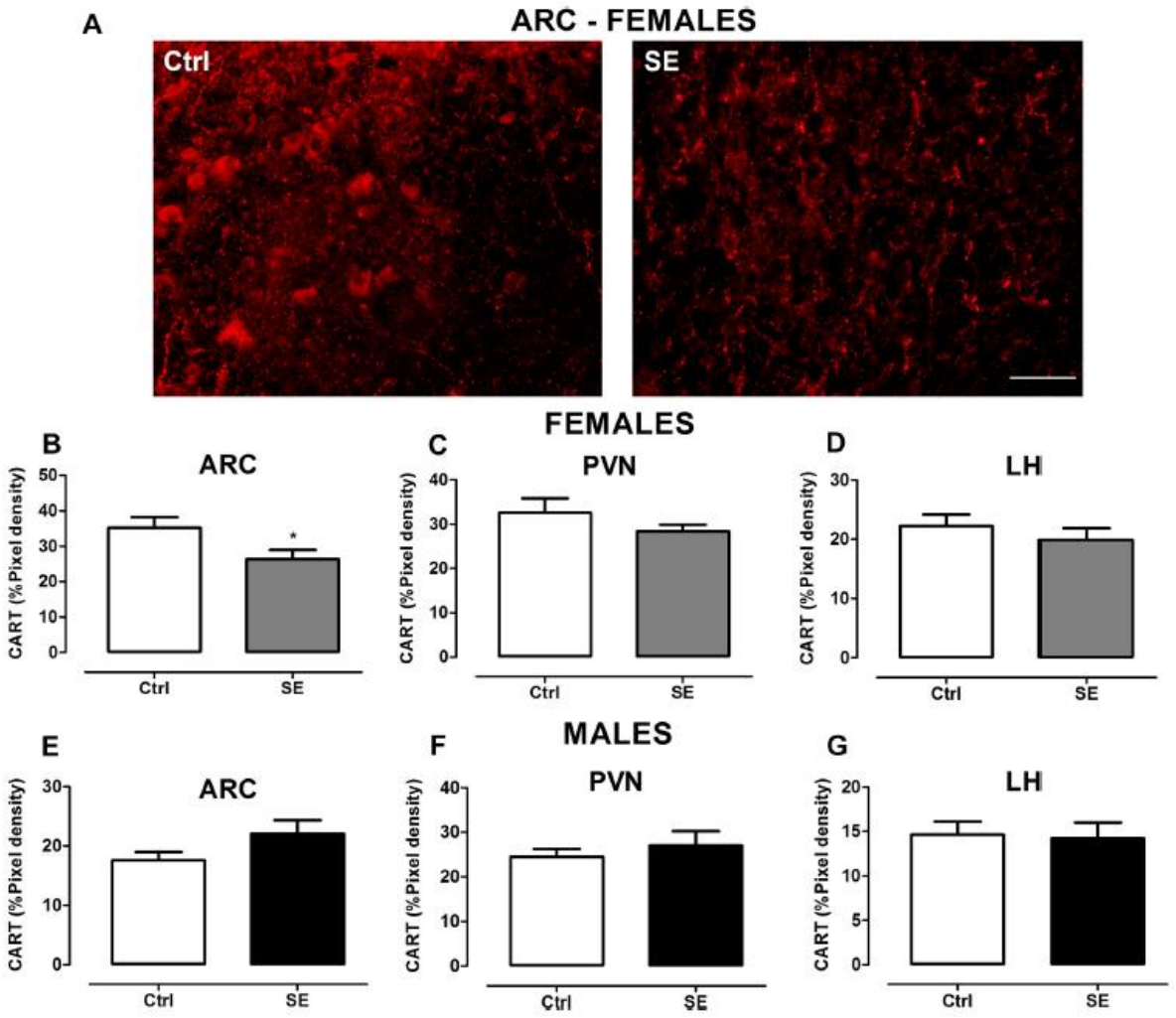

Fig. 3. Immunohistochemistry of cocaine and amphetamine-regulated transcript (CART) in the arcuate nucleus (ARC), paraventricular nucleus (PVN) and lateral hypothalamus (LH) at PND180. Immunoreaction of CART (red) in the ARC of females (A). Fiber density quantification in the ARC (B), PVN (C) and LH (D) of females. Fiber density quantification in the ARC (E), PVN (F) and LH (G) of males. Calibration bar: 50 μm. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *$p < 0.05$ vs Crtl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inflammation, which can be involved with the obese phenotype and hormone disorders previously reported. However, most changes differ between males and females, differences that, apparently, cannot be attributed to sex steroid levels. Tobacco programming did not affect serum sex hormones in males, although it caused hypoestrogenism in females, which suggests a reproductive dysfunction in spite of the fact that they presented normal estrous cycle.

Leptin positively regulates the expression of POMC, αMSH and CART (anorexigenic) and negatively the expression of AgRP and NPY (orexigenic), reducing food intake and increasing energy expenditure, consequently decreasing body mass. In a state of resistance to leptin, the energetic balance is deregulated, as indicated by hyperphagia, increased body fat and hyperleptinemia (Crujeiras et al., 2015). Although the animals of both sexes that were exposed to cigarette smoke during lactation presented a clear phenotype of central resistance to leptin at adulthood (Santos-Silva et al., 2013; Lisboa et al., 2017) we found no changes in the canonical leptin pathway (OBR, pJAK2, pSTAT3 and SOCS3) in the hypothalamus of animals exposed to cigarette smoke during lactation. The leptin regulation of hypothalamic neuropeptides is probably affected because we showed that SE males had increased NPY and reduced TRH, while SE females had reduced CART and α-MSH. Therefore, there are at least three possibilities to explain the absence of alteration in proteins expression of the leptin signaling pathway: 1) an impairment of leptin transport through the blood–brain barrier (BBB), *via* the short isoforms of the leptin receptor OBRa and OBRc

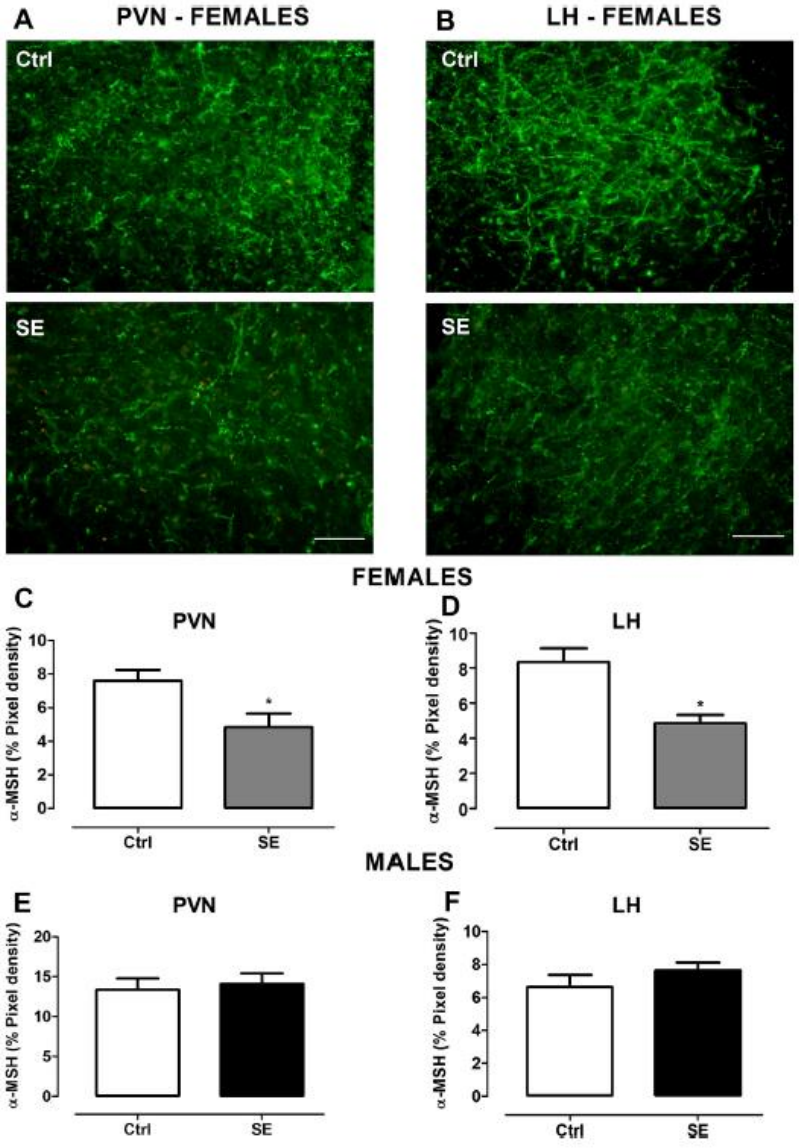

Fig. 4. Immunohistochemistry of alpha-melanocyte-stimulating hormone (a-MSH) in the paraventricular nucleus (PVN) and lateral hypothalamus (LH) at PND180. Immunoreaction of a-MSH (green) in the PVN (A) and LH (B) of females. Fiber density quantification in the PVN (C) and LH (D) of females. Fiber density quantification in the PVN (E) and LH (F) of males. Calibration bar: 50 μm. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *p < 0.05 vs Crtl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Kastin et al., 1999); besides, an increase of occludin, a membrane protein that impair the passage of leptin by the BBB, can occur (Aragonès et al., 2016); 2) a change in the alternative leptin pathway, involving the activation of phosphodiesterase 3B (PDE3B) PI3-kinase-dependent and a reduction of cAMP (Carvalheira et al., 2005); 3) one of the limitations of our study is that the current analysis was done in the total hypothalamus. Therefore, the possibility that leptin resistance is present in specific hypothalamic nuclei must be considered. In fact, studies have shown that resistance to leptin in one region of the brain can be offset by another region (Munzberg et al., 2004; Matheny et al., 2011; Rizwan et al., 2017). However, in the model of nicotine programming during lactation, protein contents of OBR, JAK2, pSTAT3 were impaired in the total hypothalamus of adult animals, findings that were associated with greater SOCS3, characterizing a condition of leptin resistance (Oliveira et al., 2010). In this study, exposure to nicotine (84 mg/kg body mass/14 days per dam) was exclusively *via* milk. Thus it is possible that the difference in leptin signaling between the study of Oliveira et al. (indirect nicotine) and the current study (direct and indirect nicotine) is most likely related to dose or route of exposure. Theoretically, in our study, the total exposure to nicotine is around 277.4 to 332.9 mg in 19 days. Comparatively, in the study of Oliveira et al., nicotine exposure resulted in a cotinine serum concentration of 20 ng/ml in the male pup at weaning, while, in the current study, the cigarette exposure results in cotinine serum concentration of 41 ng/ml in the male pups and 29 ng/ml in female pups. It is also conceivable that other components of smoke are altering the nicotine effect.

SE males showed increased NPY neuropeptide in the ARC and reduced TRH in the PVN at adulthood, which is in line with the observed hyperphagia, since NPY is orexigenic and TRH is anorexigenic (Martínez-Sánchez et al., 2014; Valdearcos et al., 2015). TRH plays an important role in the regulation of energy homeostasis, not only through an increase of thyroid hormones, which increases thermogenesis, but also through central effects on dietary behavior, reducing food intake (Lechan and Fekete, 2006). Exposure to nicotine alone during lactation is also able to increase NPY and reduce TRH in the PVN of adult offspring (Younes-Rapozo et al., 2013). Thus, the

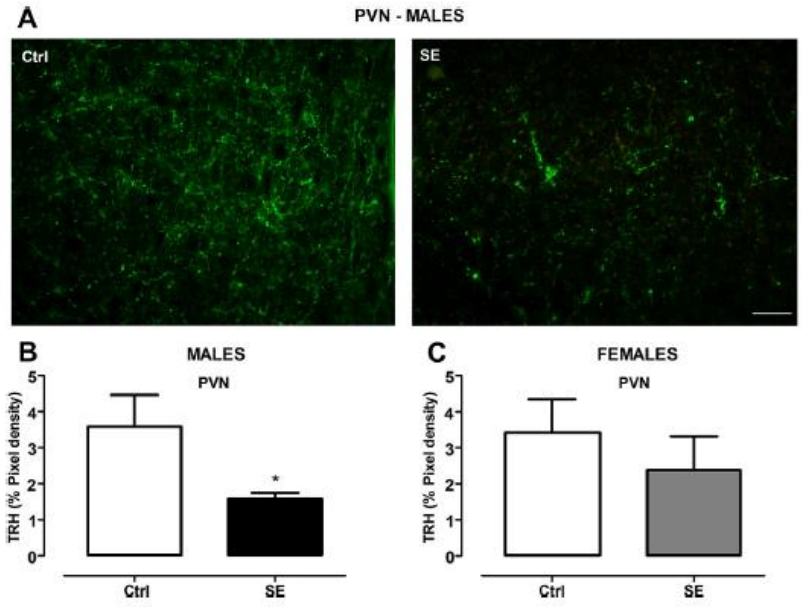

Fig. 5. Immunohistochemistry of thyrotropin-releasing hormone (TRH) in the paraventricular nucleus (PVN) at PND180. Immunoreaction of TRH (green) in the PVN of males (A). Fiber density quantification in the PVN (B) of males. Fiber density quantification in the PVN (C) of females. Calibration bar: 50 μm. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *p < 0.05 vs Crtl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

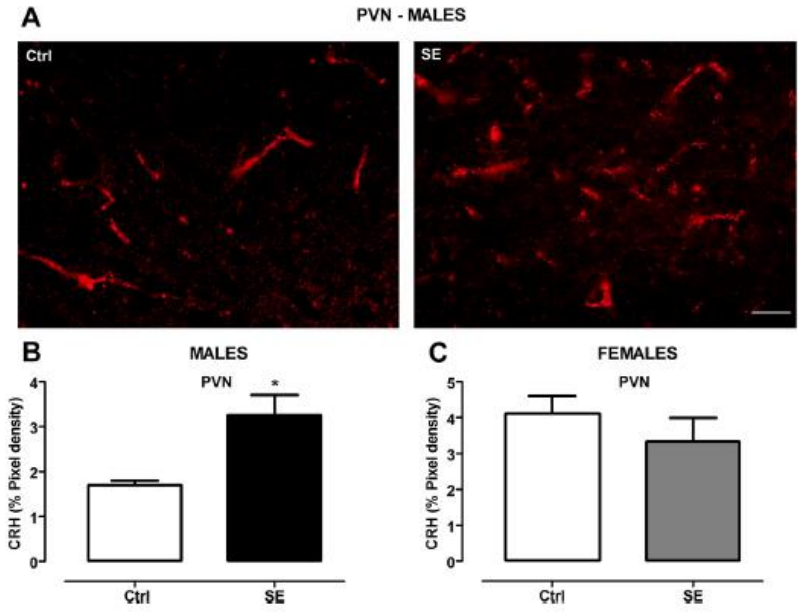

Fig. 6. Immunohistochemistry of corticotropin-releasing hormone (CRH) in the paraventricular nucleus (PVN) at PND180. Immunoreaction of CRH (red) in the PVN of males (A). Fiber density quantification in the PVN (B) of males. Fiber density quantification in the PVN (C) of females. Calibration bar: 50 μm. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *p < 0.05 vs Crtl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

presence of nicotine in the cigarette smoke is possibly responsible for these same effects on male SE animals.

As previously published, SE males showed lower plasma corticosterone, while SE females had higher plasma corticosterone (Santos-Silva et al., 2013; Lisboa et al., 2017). In the present study, SE males showed an increase of CRH in the PVN, suggesting that this tissue is responsive to the reduction of circulating corticosterone. Thus, in males, the hypocorticosteronemia may be caused by a primary adrenal deficiency. Concerning females, here we did not detect any alteration in CRH immunohistochemistry. As SE females had hypercorticosteronemia, this finding suggests a relative glucocorticoid resistance, since if negative feedback was normal, CRH should be reduced by the increased corticorterone. The measurement of adrenocorticotropic hormone (ACTH) levels would help to understand if early smoking exposure programs the malfunction of the hypothalamic–pituitary–adrenal (HPA) axis.

The adult SE females showed a reduction of α-MSH, a post-translational derivative of POMC, in the PVN and LH as well as in CART in the ARC, which may contribute to the body mass gain and hyperphagia present in these animals, since these neuropeptides inhibit food intake and increase energy expenditure (Kristensen et al., 1998; Lau and Herzog, 2014). In fact, studies have reported that nicotine withdrawal may be related to the reduction of CART and consequent increase in food intake and body mass (Levin et al., 1987; Bishop et al., 2002; Nakhate and Dandekar, 2009; Dandekar et al., 2011). Thus, the lower CART found in adult SE females in the current study, which were also observed after nicotine withdraw, may be due to smoke programming, especially due to the presence of nicotine.

Estradiol has been widely implicated in the physiological control of food intake; its decline during

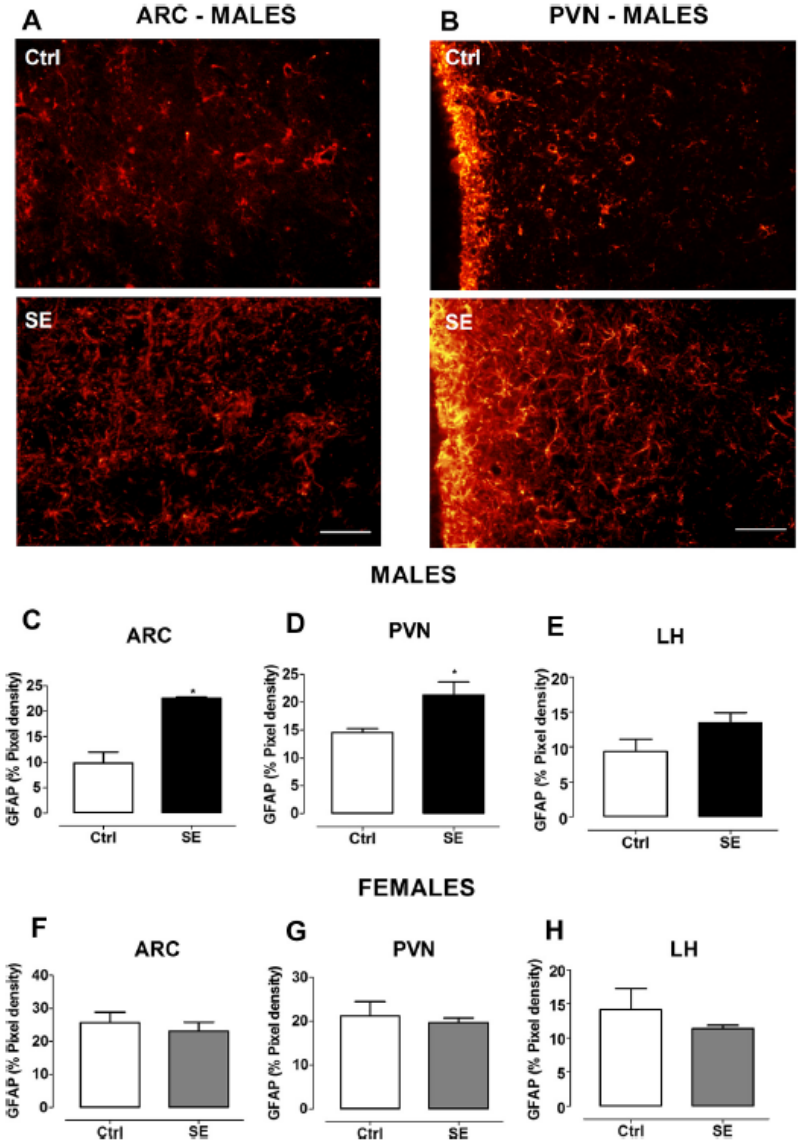

Fig. 7. Astrogliosis in animals that were directly and indirectly exposed to cigarette smoke during the breastfeeding period, revealed by glial fibrillary acidic protein (GFAP) immunohistochemistry in the arcuate nucleus (ARC), paraventricular nucleus (PVN) and lateral hypothalamus at PND180. Immunoreaction of GFAP (red) in the ARC (A) and PVN (B) of males. Fiber density quantification in the ARC (C), PVN (D) and LH (E) of males. Fiber density quantification in the ARC (F), PVN (G) and LH (H) of females. Calibration bar: 50 μm. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *p < 0.05 vs Crtl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

menopause is related to obesity (Pfaff and Keiner, 1973) and ovariectomy is associated with hyperphagia (Butera, 2010; Dandekar et al., 2012). In addition, estradiol treatment of ovariectomized rats increased CART expression and, consequently, contributed to the anorectic effect (McElroy and Wade, 1987). Thus, the reduction of serum estradiol levels in SE females can contribute to the lowering of CART expression as well as to the hyperphagic state.

Many studies have already reported the occurrence of hypothalamic inflammation in diet-induced obesity models, in which the process of central inflammation precedes systemic inflammation (Waise et al., 2015). Hypothalamic inflammation favors leptin resistance and causes structural changes in the hypothalamic circuits, compromising the energy balance (De Git and Adan, 2015; Younes-Rapozo et al., 2015a). In addition, our group has already demonstrated the presence of hypothalamic inflammation in adult animals of different programming models of obesity associated with leptin resistance (Younes-Rapozo et al., 2015a; Younes-Rapozo et al., 2015b).

Chan, et al. demonstrated that gestational smoke exposure is capable of increasing IL6 and toll like receptor (TLR) 4 expressions in the pups' brains at PND90 (Chan et al., 2016). In another study, where dams were exposed to cigarette smoke during mating, gestation and lactation, the pups had, at weaning, increased nitric oxide synthase (iNOS) in the brain, indicating the presence of inflammation and/or oxidative stress (Chen et al., 2018). Also, in the present work, in which the exposure to tobacco smoke occurred only during lactation, through the milk (indirect exposure) plus inhalation (direct exposure), we evidenced gliosis in the hypothalamus. In our experimental model, this inflammation is possibly associated with hyperleptinemia. Previously, it was shown that exposure to nicotine alone during lactation is able to program male rat offspring to hypothalamic gliosis (Younes-Rapozo et al., 2015a). Here, we demonstrated that, like nicotine alone, the neonatal exposure to cigarette smoke promotes the increase in GFAP and IL6 in the male rat offspring. Therefore, the hypothalamic gliosis found in SE males can be mainly caused by nicotine. Interestingly, this alteration was not observed in SE females. Sexual dimorphism in the inflammatory response occurs because astrocytes express estrogen receptors (Garcia-Segura et al., 1996; Melcangi et al., 2001) and

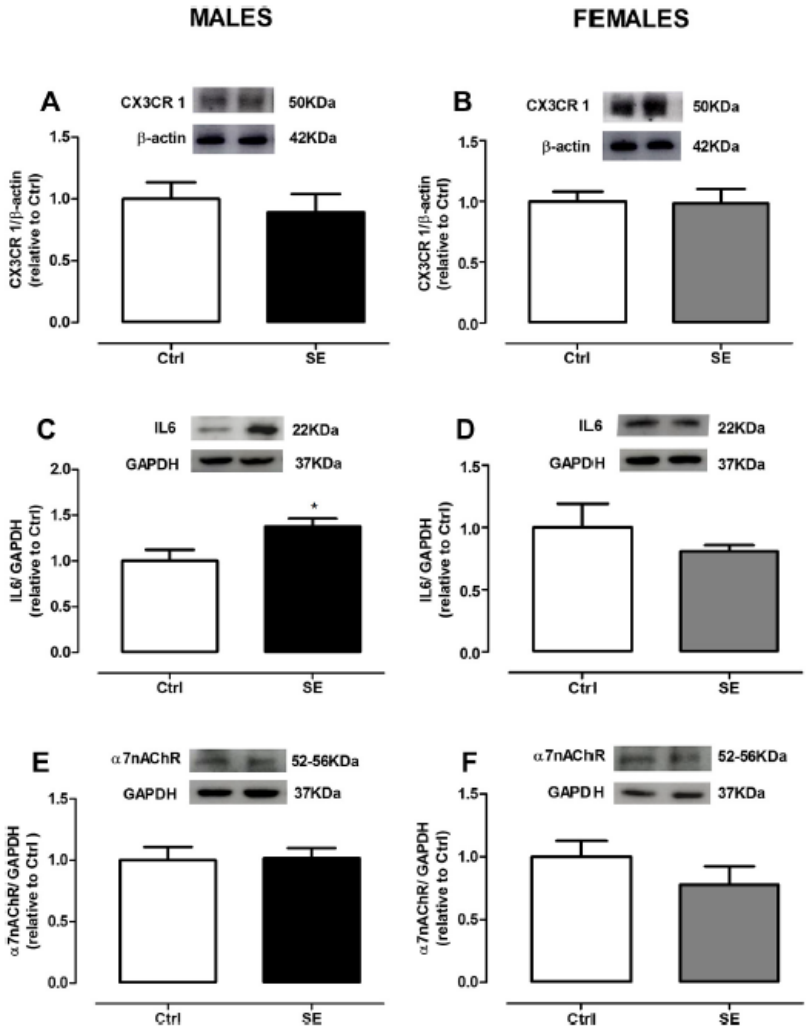

Fig. 8. CX3R1, IL6 and α7nAChR in the hypothalamus of PND180 animals that were directly and indirectly exposed to cigarette smoke during the breastfeeding period. CX3R1 protein contents in the hypothalamus of males (A) and females (B). IL6 protein contents in the hypothalamus of males (C) and females (D). α7nAChR protein contents in the hypothalamus of males (E) and females (F). Representative blots of the proteins are shown beside the graphs. β-Actin content was used as control loading. Values are expressed as relative (%) to the control group. Ctrl: control group; SE: smoke-exposed group. Results are expressed as mean ± SEM, five to eight/group. *p < 0.05 vs Crtl.

progesterone receptors (Garcia-Ovejero et al., 2005; Wang et al., 2015); for this reason, women have a protection regarding the development of hypothalamic gliosis. Even more important than the effects of sex hormones alterations on the central inflammation in the present model are possibly the differences in corticosterone levels, a well-known anti-inflammatory hormone. The hypocorticosteronemia observed in SE males and the hypercorticosteronemia observed in SE females (Santos-Silva et al., 2013; Lisboa et al., 2017) may explain why males had hypothalamic inflammation, while females seemed to be protected.

Some considerations regarding the present experimental design deserve attention. Nicotine, *via* cigarette smoking, is the drug most used during pregnancy, causing several deleterious effects to the fetus and to the newborn. Many women who quit smoking during pregnancy relapse during breastfeeding. Maternal active smoking during this period is harmful for the lactating mothers, but it is also for the infants who are involuntarily exposed to cigarette components through second-hand smoking and contamination of breast milk. In this context, the current animal model is relevant since it allows us to have some insights regarding both the direct (environmental) and the indirect (breast milk) effects of exposure to components of tobacco smoke.

In summary, taken together, our present findings evidence that early exposure to tobacco smoke is deleterious to the hypothalamic circuitry later in the life of the progeny, inducing changes in energy homeostasis that favor both hyperphagia and obesity development. Based on previously published data, at least in adult males, the observed alterations can be attributed to a nicotine programming effect.

ADDITIONAL INFORMATION

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COMPETING INTERESTS

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

AUTHOR CONTRIBUTIONS

- Concept and design: TCP; EGM; PCL.
- Animal treatment, collection of samples and measurements: TCP; VYR; EO; PNS; VSTR; MAT; AST.
- Analysis and interpretation of data: all authors.
- Drafting and/or revising the article critically for important intellectual content: TCP; EO; EGM; PCL; ACM.

ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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3.3 **Artigo 3**: **Nicotine Exposure During Breastfeeding Reduces Sympathetic Activity in Brown Adipose Tissue and Increases in White Adipose Tissue in Adult Rats: Sex-related Differences (artigo publicado)**

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Nicotine exposure during breastfeeding reduces sympathetic activity in brown adipose tissue and increases in white adipose tissue in adult rats: Sex-related differences

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ABSTRACT

Nicotine transfer via breast milk induces obesity in the adult offspring. We hypothesize that sympathetic nervous system (SNS) activity, brown adipose tissue (BAT) thermogenesis and white adipose tissue (WAT) lipogenesis/adipogenesis are altered in adult rats that were exposed to nicotine exclusively during the breastfeeding period. Lactating Wistar rats were separated into two groups: nicotine (NIC), dams implanted with osmotic minipumps containing 6 mg/kg of nicotine at postnatal day (PN) 2; control, dams were implanted with saline-containing minipumps. Euthanasia occurred at PN120 or PN180. NIC offspring had lower BAT SNS activity and higher BAT lipid content. NIC males showed lower UCP1, β3-AR and CPT1a, while NIC females showed lower UCP1, TRα1, CPT1a, suggesting lower thermogenesis. NIC males showed higher WAT SNS activity, WAT β3-AR, adrenal catecholamine, FAS, PPARγ and adipocytes area, while NIC females showed higher ACC, FAS, CEBPβ and PPARγ. These findings indicate increased lipogenesis/adipogenesis in both sexes, with a possible compensatory sympathetic activated-lipolysis in males. NIC males had higher hypothalamic pAMPK/AMPK, explaining the lower BAT sympathetic activity. Neonatal nicotine exposure reduces BAT SNS activity and thermogenesis, and, only in males, increases WAT adipogenesis/lipogenesis, despite higher WAT SNS activity. These alterations can be associated with obesogenesis in this programming model.

1. Introduction

Obesity prevalence is increasing worldwide (Engin, 2017). Different conditions may lead to obesity and other related chronic diseases, such as high calorie intake, physical inactivity and genetic conditions (Rao et al., 2019; Penninx and Lange, 2018). According to clinical and pre-clinical data, environmental and nutritional insults during fetal or perinatal life can program epigenetic changes that contribute to the onset of different metabolic disorders later in life, such as obesity, dyslipidemias, type 2 diabetes mellitus, hypertension, hormonal and behavioral changes (Fraga-Marques et al., 2010; Hanley et al., 2010; Lisboa et al., 2017; Almeida et al., 2019; Krzeczkowski et al., 2019). In this sense, both experimental and epidemiological studies have shown that smoking during breastfeeding may increase the likelihood of the offspring developing obesity as adults (Lisboa et al., 2017; Mizutani et al., 2007; Oken et al., 2008; Santos-Silva et al., 2013). Some women stop smoking during gestation but resume it during breastfeeding, believing it does not affect their babies (Hannöver et al., 2008; McBride and Pirie, 1990). However, nicotine, the main additive molecule of the cigarette, is transferred by breast milk and is capable of promoting several short- and long-term adverse effects. In rodents, maternal nicotine exposure exclusively during the lactation period programs for higher body and visceral fat mass, hyperleptinemia, hyperinsulinemia, hypercorticosteronemia and hypothyroidism in the male offspring (Oliveira et al., 2009; de Oliveira et al., 2010). The female offspring in this programming model show no changes in body mass or plasma hormones (Pinheiro et al., 2011). Our group has previously shown that exposure to cigarette smoke exclusively during lactation increased visceral fat mass, food intake and circulating leptin in the progeny of both sexes, while hyperthyroidism was observed only in males (higher TSH, T4 and T3) and increased serum T4 was observed only in females (Santos-Silva et al., 2013; Lisboa et al., 2017).

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A study in rats, in which nicotine was administered subcutaneously (1.0 mg/kg body mass, twice daily) during both pregnancy and lactation reasserted the relationship between early exposure to nicotine and the onset of late-occurring metabolic disorders (Fan et al., 2016). Perinatal exposure to nicotine induced an increase of body mass and changes in BAT morphology and function in the adult progeny: six-month-old male offspring exhibited reduced mRNA expression of some thermogenesis-related genes (PRDM16, CIDEA, PGC1α, UCP1 and CPT2), BAT whitening and alterations of mitochondrial structure. The adult female progeny was not evaluated in the aforementioned study. Recently, in the programming model of smoke exposure restricted to the breastfeeding period, we demonstrated an increase in BAT mass and a decrease in BAT SNS and thermogenesis biomarkers in both adult males and females (Peixoto et al., 2018). Since cigarettes have more than 7,000 compounds (WHO, 2019; Rodgman and Perfetti, 2013) and since some of the observed alterations seem to differ between nicotine-only exposure and tobacco-smoke exposure, it is relevant to evaluate whether nicotine-only exposure also has effects on BAT SNS activity and thermogenesis.

Both BAT and white adipose tissue (WAT) are innervated by the SNS (Contreras et al., 2015). In general, SNS responses are regionalized and do not follow a simple pattern in obese individuals (Lambert et al., 2019). In obesity, the SNS increases lipolysis in the WAT in response to adipocyte hypertrophy, providing more free fatty acids (FFA), which contributes to fat accumulation in other tissues, lipotoxicity and insulin resistance (Li et al., 2018). Lipolysis triggered by SNS depends on adrenergic receptors in adipocytes (Langin, 2006). Concerning BAT, obesity contributes to reduction of SNS activity, compromising its thermogenic activity, thereby favoring even more the accumulation of fat and body mass increase (Trayhurn, 2017; Waldén et al., 2012). Given these observations, the development of new strategies for the treatment of obesity and metabolic disorders in adult humans is being based on the intensification and/or restoration of BAT thermogenesis, contributing to increased energy expenditure, reduction of body mass and improvement of metabolic status (Feldmann et al., 2009; Luijten et al., 2019b; Saito, 2013; Soler-Vázquez et al., 2018).

BAT is responsible for the dissipation of energy via the uncoupling protein 1 (UCP1), resulting in increased fatty acid oxidation and heat production (Cannon, 2004; Fenzl and Kiefer, 2014). BAT thermogenesis is controlled by: 1) activation of the beta 3-adrenergic receptor (β3-AR) (Contreras et al., 2015; Morrison et al., 2014); 2) thyroid hormones, which increase both UCP1 levels and the adrenergic sympathetic response (Martínez-Sánchez et al., 2014); 3) proliferator-activated γ receptor coactivator 1α (PGC-1α), which regulates mitochondrial biogenesis, the Krebs cycle and the beta-oxidation of fatty acids (Fernandez-Marcos and Auwerx, 2011); 4) carnitine palmitoyltransferase 1 (CPT1A), which increases mitochondrial uptake and oxidation of long chain fatty acids (Bonnefont et al., 2004); 5) AMP-activated protein kinase (AMPK), which is positively associated with UCP1 activity as well as is involved in the maintenance of thermogenesis (Desjardins and Steinberg, 2018). The central innervation of BAT comes from brain autonomic centers, including the ventromedial (VMH) and paraventricular (PVN) nuclei of the hypothalamus (White et al., 2016). In the PVN, the melanocortin 4 receptor (MC4R) stimulates SNS activity (Berglund et al., 2014), while the activation of AMPK in the VMH reduces sympathetic activity and, consequently, thermogenesis (López et al., 2016).

As previously described in the literature, nicotine exposure throughout gestation and lactation has a negative impact on BAT thermogenesis in the adult male offspring (Fan et al., 2016) but not in females. Concerning nicotine-only exposure, some aspects of its effects remain unclear, especially those regarding the window of exposure and the fact that the outcomes are reported only in male offspring. Furthermore, other aspects of BAT thermogenesis have not been not studied thus far. Our hypothesis is that nicotine exposure through milk is as harmful as nicotine exposure via placenta + milk, possibly more so. Therefore, the current study was designed to evaluate the long-term effects of nicotine-only exposure during the breastfeeding period on the BAT and WAT function in both male and female offspring at distinct ages (PN120 and PN180). In this programming model, we investigated the sympathetic nerve activity (BAT and WAT), morphological structure of BAT and WAT, protein content of different thermogenic biomarkers (thyroid hormone and adrenergic receptors, mitochondrial biogenesis and fatty acid oxidation), WAT biomarkers linked to catecholamine sensitivity (lipolysis), adipogenesis (PPARγ, CEBPβ) and lipogenesis (ACC and FAS), total catecholamine in the adrenal gland as well as hypothalamic control of the SNS for BAT.

2. Materials and methods

2.1. Ethics, animals and experimental groups

The Institutional Ethical Committee for the use of laboratory animals of the Biology Institute of the State University of Rio de Janeiro approved all experimental procedures (CEUA/007/2017). Experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Wistar rats were housed under controlled temperature (23/24 °C), photoperiod of 12 h (light/dark cycle), with water and food *ad libitum*. For mating, virgin adult female rats were placed with males in a 2 to 1 ratio for one week. Each pregnant rat was housed in individual cages until delivery. At birth, which was considered as the first postnatal day (PN1), litters were adjusted to 6 pups for each dam (3 females and 3 males). At PN2, dams were randomly separated into two groups: 1) Nicotine-exposed group (NIC) - 7 lactating rats were implanted with osmotic minipumps (Alzet, 2ML2, CA, USA) that released 6 mg/kg of nicotine per day for 14 days (Oliveira et al., 2009; Pinheiro et al., 2011). Our model produces plasma cotinine (main nicotine metabolite) concentrations of approximately 25 ng/mL, similar to those found in typical smokers (Lichtensteiger et al., 1988); 2) Control group (C) - 7 lactating rats were implanted with osmotic minipumps containing only saline solution, which was released for the same period indicated above. To allow for the subcutaneous insertion of the pumps, dams were lightly anesthetized with thiopental, and a 3 × 6 cm area on the back was shaved. This surgery is extremely fast, taking about 2–3 min. There was no need for specific medical care after the implantation since there was no infection. Pump implantation occurred at PN2 because 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 (manufacturer's recommendation). The incision was closed and dams were permitted to recover in their home cages.

From weaning (PN21) to euthanasia (at PN120 or PN180), male and female pups were kept separately in different cages (3 per sex/cage). From PN90 to PN180, the estrous cycle was analyzed. Both female groups (C and NIC) had regular 4-5-day estrous cycles and were euthanatized during diestrous.

2.2. BAT and WAT sympathetic nervous system (SNS) activity

One rat of each sex per litter per group was anesthetized with a 2:1 solution of xylazine (Xilazin®, 100 mg/kg BM) and ketamine (Cetamin®, 50 mg/kg BM) after 12 h of fasting. Then, animals were evaluated for *in vivo* analysis of the sympathetic nerve of the interscapular BAT (iBAT; PN120 and PN180) and for *in vivo* analysis of the sympathetic nerve of the WAT (PN180), located in the lumbar plexus, near the retroperitoneal fat. Animals were placed inside a faraday cage to avoid electromagnetic interference; the nerves were placed on a pair of platinum hook electrodes connected to an electronic device (Bio-Amplifier, Insight®, Ribeirão Preto, SP, Brazil) to record the electrical signals. To avoid dehydration, the nerve was covered with mineral oil. Nerve activity was amplified (10,000 ×) and filtered (cut-off: 60 kHz). A 5-min

period of stabilization and a 10-min reading period were used. The average number of spikes per 10-s time window was noted. The background noise level was determined in a nerve segment. Results were analyzed using the PowerLab data acquisition system (8SP; AD Instruments, New South Wales, Australia) (Peixoto et al., 2018; Venci et al., 2018).

2.3. Euthanasia and tissue collection

After the *in vivo* measurement of BAT SNS activity (PN120 and PN180) and WAT SNS activity (PN180), animals were euthanized by cardiac puncture and the tissues of interest were collected. The iBAT was dissected, weighed and prepared for morphological (kept in paraformaldehyde) and molecular studies (kept at −80 °C). The visceral fat compartments (mesenteric, gonadal and retroperitoneal deposits) were collected to obtain total visceral fat mass (VFM). The retroperitoneal deposit was prepared for morphological (kept in paraformaldehyde) and molecular studies (kept at −80 °C). The whole brain was removed and stored at −80 °C until dissection of the nuclei of interest. Blood samples were centrifuged (1,500×*g* for 20 min at 4 °C) to obtain plasma and kept at −20 °C. Leptin levels were determined by a rat ELISA kit (Millipore, MO, USA).

2.4. BAT and WAT morphological analysis

Samples of both adipose tissues were fixed in paraformaldehyde (4%) and embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) and sectioned at 5 μm thickness. Sections were fixed onto glass slides (3/slide) for hematoxylin/eosin staining. Digital images were randomly acquired (TIFF format, 36-bit color, 1,360 × 1,024 pixels) from the sections using an Olympus DP71 camera and an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan). At least 10 randomly-selected photomicrographs per animal were analyzed with the software Image-Pro plus 5.0 (Media Cybernetics, Silver Spring, MD, USA).

In the BAT, the digital images of the droplets were analyzed and the areas of the droplets were used for the measurement of the percentage of vesicles per tissue area. Images were classified according to the size and number of vesicles that were present. In the WAT, a total of 100 adipocytes per animal (n = 10 per photomicrographs) were used for the determination of adipocyte area (μm). The digital images were used to assess the area of the droplets.

2.5. Isolation of the hypothalamic nuclei

To obtain the coronal sections of the brain, a cryostat (Hyrax C52, Zeiss, Germany) was used. For the isolation of the paraventricular (PVN, Bregma −0.6 to −2.1 mm) and ventromedial (VMH, Bregma −2.1 to −3.6 mm) nuclei of the hypothalamus, the coordinates described in Paxinos and Watson stereotaxic atlas were used (Paxinos and Watson, 1998). The samples were then frozen at −80 °C for use in the western blotting.

2.6. Western blotting analysis

Protein expression in the BAT, WAT, PVN and VMH were evaluated by Western blot. For protein extraction, 7 samples of each tissue were homogenized. BAT and WAT, which were frozen in liquid nitrogen, were subjected to maceration in specific protein extraction buffer (TPER Tissue Protein Extraction). For the PVN and VMH the RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF] was used. These buffers contained a protease inhibitor cocktail (F. Hoffmann-La Roche Ltd., Basel, CH). BAT and WAT homogenates were centrifuged (12,851×*g*, 4 °C, 30 min) and then the intermediate phases were collected. The PVN and VMH were sonicated (two pulses of 10 s with 40% amplitude, intercalated by 15 s off). Protein concentration in the supernatants was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, CA, USA). Homogenates were analyzed by SDS-PAGE using 20 μg (BAT and WAT) or 10 μg (PVN and VMH) total protein. Samples were transferred onto PVDF membranes (Hybond ECL; Amersham Pharmacia Biotech, London, UK). All membranes (BAT, WAT, PVN, ARC and VMH were incubated with Tris-buffered saline (TBS) containing 5% albumin for 45 min. Subsequently, BAT membranes were incubated with specific primary antibodies: anti-UCP1 (1:500, Sigma-Aldrich, Invitrogen Corporation CA, USA), anti-β3-AR (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-TRβ1 and anti-TRα1 (1:500, Abcam, MA, UK), anti-PGC1α and CPT1a (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-AMPKα and anti-phospho-AMPKα (1:500, Cell Signaling Technology, Inc., MA, USA) overnight at 4 °C. WAT membranes were incubated with specific primary antibodies: anti-ACC and anti-FAS (1:500, Cell Signaling Technology, Inc., MA, USA), anti-CEBPβ, anti-PAPRγ and anti-β3-AR (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4 °C. PVN membranes were incubated with anti-AMPKα, anti-phospho-AMPKα (1:500, Cell Signaling Technology, Inc., MA, USA) overnight at 4 °C. VMH membranes were incubated with anti-MC4R (1:500, Abcam, MA, UK) overnight at 4 °C.

Either anti-β−actin (1:500) or GAPDH (1:1000) primary antibodies (depending on protocol) were used as internal control for each membrane (Sigma-Aldrich, Invitrogen Corporation CA, USA). Membranes were washed 3 times with Tween-TBS (0.1%) and then incubated for 1 h with the appropriate concentration of the secondary antibody (1:5000, 1:7000 or 1:10000) conjugated with biotin (anti-rabbit, anti-mouse or anti-goat Sigma-Aldrich, Invitrogen Corporation CA, USA) at room temperature. Membranes were then washed 3 times with Tween-TBS (0.1%), which was followed by the incubation with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA, USA). Protein bands were visualized by chemiluminescence (Kit ECL plus, Amersham Biosciences, London, UK) followed by exposure to Image Quant LAS (GE Healthcare, Buckinghamshire, UK). The bands area and density were quantified by Image J software (Wayne Rasband National Institute of Health, MA, USA) and normalized for β-actin or GAPDH. Results were expressed as relative (%) to the control group (C).

2.7. Determination of the total catecholamine

The right adrenal gland was used for the measurements of total catecholamine levels through the trihydroxyindole method, using epinephrine as a standard (Conceição et al., 2013). Briefly, adrenal glands were homogenized with 10% acetic acid; homogenate was centrifuged (1,120×*g*, 5 min, 4 °C). Then, 50 μL of epinephrine or the supernatant was mixed with 250 μL of 0.5 M phosphate buffer, pH 7.0, and 25 μL of 0.5% potassium ferricyanate and incubated (20 min, ice bath). The reaction was stopped with 500 μL of 60 mg/mL ascorbic acid and 5 N NaOH (1:19 ratio), and the samples were diluted with 2 mL of distilled water. The fluorescence was determined at 420 nm for excitation and 510 nm for emission (Hidex, Turku, FI).

2.8. Statistical analysis

Data were analyzed with the statistical program GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA) and are expressed as means ± standard error of the mean (SEM). Comparisons between C and NIC groups were performed using Student's unpaired *t*-test since the programming effects in male and female rats were separately evaluated. Differences were considered significant when $p < 0.05$.

Table 1
Long-term effects of nicotine-only exposure during breastfeeding on biometric parameters and leptinemia of offspring at different ages.

	PN120			
	MALE		FEMALE	
	C	NIC	C	NIC
Body mass (g)	486 ± 14	473 ± 7	257 ± 6	251 ± 4
VFM (g)/100 g BM	4.6 ± 0.3	5.3 ± 0.3	4.3 ± 0.3	4.1 ± 0.3
iBAT mass (g)/100 g BM	0.060 ± 0.004	0.080 ± 0.004*	0.090 ± 0.005	0.090 ± 0.001
Leptin (ng/mL)	10.4 ± 1.6	9.4 ± 1.2	3.7 ± 0.4	2.9 ± 0.2
	PN180			
	MALE		**FEMALE**	
	C	**NIC**	**C**	**NIC**
Body mass (g)	542 ± 7	574 ± 8*	294 ± 4	299 ± 4
VFM (g)/100 g BM	5.9 ± 0.2	6.7 ± 0.3*	5.6 ± 0.3	6.2 ± 0.3
iBAT mass (g)/100 g BM	0.080 ± 0.004	0.100 ± 0.004*	0.090 ± 0.004	0.100 ± 0.010
Leptin (ng/mL)	7.0 ± 0.9	10.5 ± 1.3*	5.0 ± 1.2	7.8 ± 1.3

C: control group; **NIC:** nicotine group. **VFM:** Visceral fat mass; **BM:** Body mass; **iBAT:** Interscapular brown adipose tissue. Values represent means ± SEM, n = 7/group. *p < 0.05 *vs* **C**.

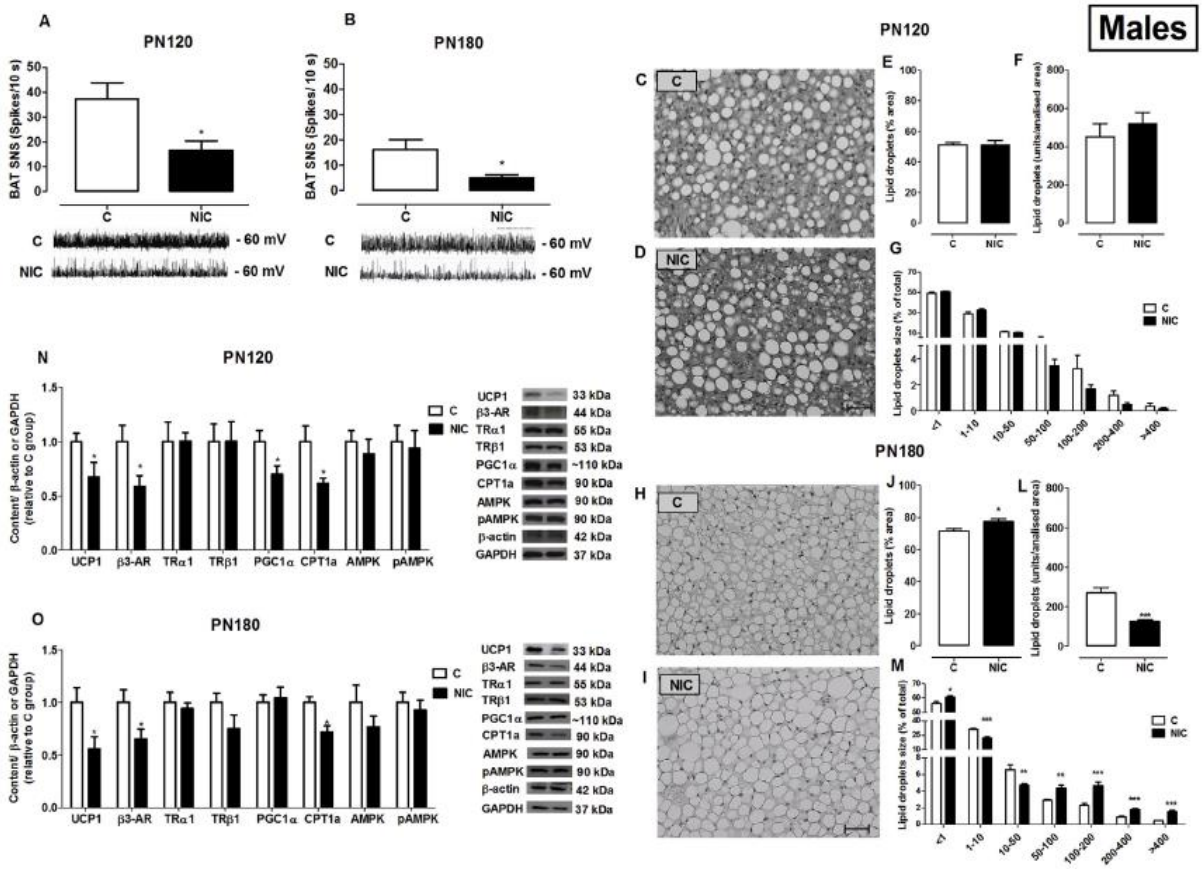

Fig. 1. Effect of nicotine-only exposure during breastfeeding on BAT in male offspring. BAT SNS at PN120 (**A**) and PN180 (**B**) rat offspring. BAT morphology (lipid droples: % area, units per area and size) at PN120 (**C, D, E, F and G**) and PN180 (**H, I, J, L and M**). BAT biomarkers (UCP1, β3-AR, TRα1, TRβ1, PGC1α, CPT1a, AMPK and pAMPK) at PN120 (**N**) and PN180 (**O**). **C:** control group; **NIC:** nicotine group. Results are expressed as mean ± SEM; *p < 0.05; **p < 0.01, n = 7.

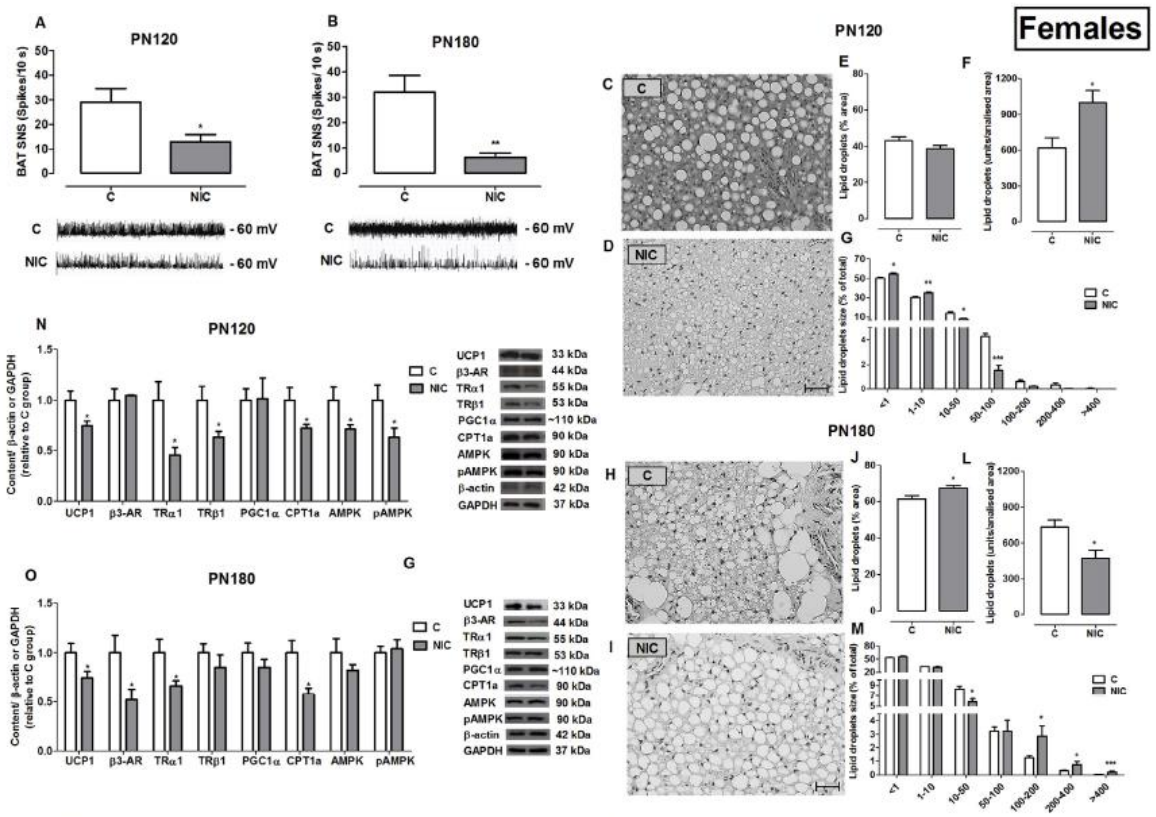

Fig. 2. Effect of nicotine-only exposure during breastfeeding on BAT in female offspring. BAT SNS at PN120 (**A**) and PN180 (**B**) rat offspring. BAT morphology (lipid droplets: % area, units per area and size) at PN120 (**C, D, E, F and G**) and PN180 (**H, I, J, L and M**). BAT biomarkers (UCP1, β3-AR, TRα1, TRβ1, PGC1α, CPT1a, AMPK and pAMPK) at PN120 (**N**) and PN180 (**O**). **C**: control group; **NIC**: nicotine group. Results are expressed as mean ± SEM; *p < 0.05; **p < 0.01, n = 7.

3. Results

3.1. Body mass, fat deposit and plasma leptin

As shown in Table 1, at PN120, NIC animals of both sexes had no change in body mass and VFM. Only NIC males had higher iBAT mass at PN120 (+33% *vs* C, p < 0.05). No change in plasma leptin levels was detected. At PN180, NIC males had higher body mass (+6% *vs* C, p < 0.05), VFM (+14% *vs* C, p < 0.05), iBAT mass (+17% *vs* C, p < 0.05) and plasma leptin (+49% *vs* C, p < 0.05), while NIC females showed no change of these parameters (Table 1).

3.2. In vivo BAT SNS, morphology and function

NIC animals had lower SNS activity at the basal condition in the BAT at PN120 (−51% *vs* C males; −56% *vs* C females, p < 0.05, Figs. 1A and 2A) and at PN180 (−69% *vs* C males; −80% *vs* C females, p < 0.05, Figs. 1B and 2B). As depicted in Figs. 1E and 2E, at PN120, NIC animals had no change in the percentage of lipid droplet in BAT total area. Only NIC females showed an increase of 60% in the lipid unit per area, suggesting more lipid droplets in BAT (p < 0.05, Fig. 2F and D). Fig. 2G shows that NIC females have a smaller lipid sizes profile in BAT.

At PN180, NIC animals showed higher percentage of lipid droplet in BAT total area (+8.5% *vs* C males; +9.5% *vs* C females, p < 0.05, Figs. 1J and 2J). NIC groups had lower lipid unit per area (−53% *vs* C males; −36% *vs* C females, p < 0.05, Fig. 1L and 2L) and larger lipid sizes profile (p < 0.05, Fig 1M and 2M), suggesting that the increase in the percentage of lipid is due to hypertrophied adipocytes.

Regarding BAT biomarkers of thermogenesis at PN120, NIC animals showed lower protein content of UCP1 (−33% *vs* C males; −25% *vs* C females, p < 0.05, Fig. 1N and 2N) and CPT1a (−39% *vs* C males; −28% *vs* C females, p < 0.05, Fig.1N and 2N). Besides, NIC males also showed lower protein content of β3-AR (−41% *vs* C, p < 0.05) and PGC1α (−30% *vs* C, p < 0.05), while NIC females showed lower protein content of TRα1 (−55% *vs* C, p < 0.05), TRβ1 (−36% *vs* C, p < 0.05), AMPK (−28% *vs* C, p < 0.05) and pAMPK (−36% *vs* C, p < 0.05), as shown in Fig.1N and 2N).

At PN180, NIC animals had lower protein content of UCP1 (−43% *vs* C males; −26% *vs* C females, p < 0.05, Fig. 1O and 2O), β3-AR (−35% *vs* C males; −48% *vs* C females, p < 0.05, Figures 1O and 2O) and CPT1a (−28% *vs* C males; −42% *vs* C females, p < 0.05, Fig. 1O and 2O). In addition, NIC females exhibited lower TRα1 protein content (−34% *vs* C, Fig. 2O).

3.3. In vivo WAT SNS, morphology and function

At PN120, NIC animals had no change in the adipocytes area in the WAT (Figs. 3C and 4C), while, at PN180, these animals showed higher WAT adipocytes area (+27% *vs* C males; +30% *vs* C females,

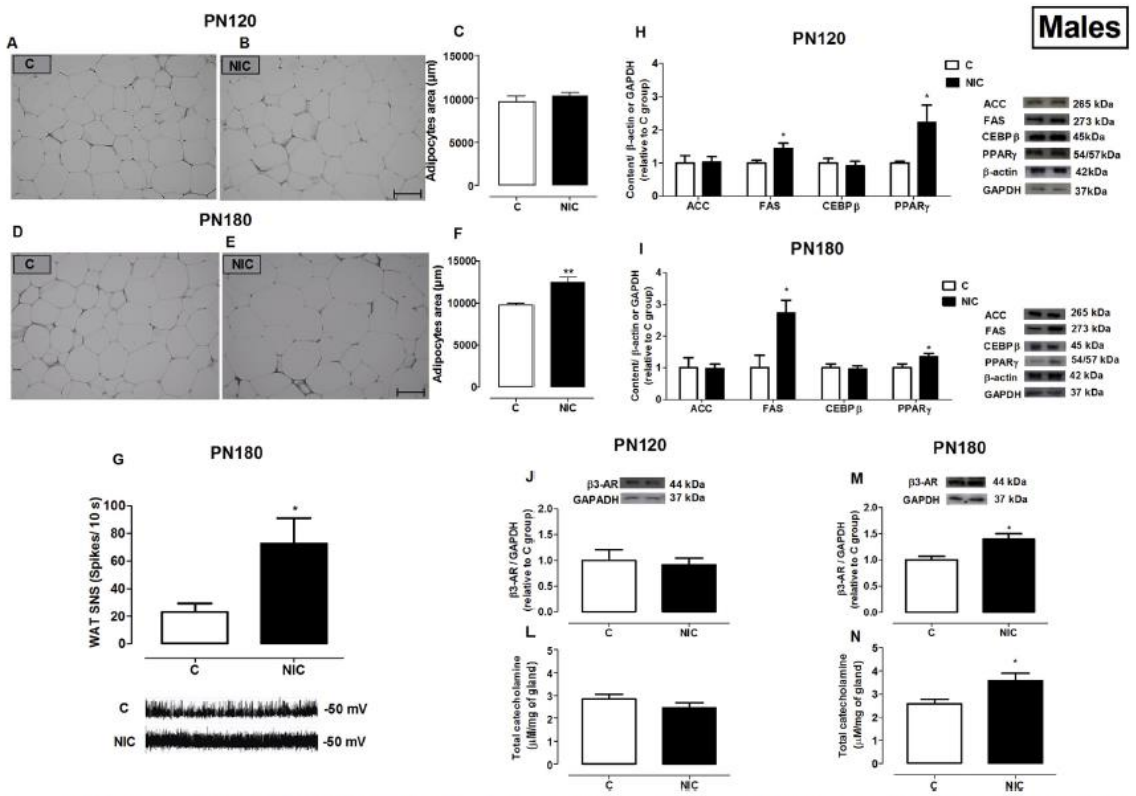

Fig. 3. Effect of nicotine-only exposure during breastfeeding on WAT in male offspring. Morphology and adipocytes area at PN120 (**A, B and C**) and PN180 (**D, E and F**) rat offspring. WAT SNS at PN180 (G). Lipogenic and adipogenic biomarkers (ACC, FAS, CEBPβ and PPARγ) at PN120 (**H**) and N180 (**I**). β3-AR protein contents at PN120 (**J**) and PN180 (**M**). Adrenal total catecholamine content at PN120 (**L**) and PN180 (**N**). **C**: control group; **NIC**: nicotine group. Results are expressed as mean ± SEM; *$p < 0.05$; **$p < 0.01$, $n = 7$.

$p < 0.05$, Figs. 3F and 4F).

At PN180, only NIC males showed an increase of SNS activity at the basal condition in the WAT (+2-fold, $p < 0.05$, Fig. 3G). Regarding WAT lipogenic and adipogenic biomarkers, at PN120, NIC animals showed higher protein content of FAS (+43% *vs* C males; +47% *vs* C females, $p < 0.05$, Figs. 3H and 4H) and PPARγ (+1.2-fold *vs* C males; +95% *vs* C females, $p < 0.05$, Figs. 3H and 4H). No change was found in ACC, CEBPβ (Figs. 3H and 4H), β3-AR or total catecholamine (Fig. 3J, L, 4J and 4L).

At PN180, NIC males showed higher protein content of FAS (+1.7-fold *vs* C, $p < 0.05$, Fig. 3I), PPARγ (+36% *vs* C, $p < 0.05$, Fig. 3I) and β3-AR (+40% *vs* C, $p < 0.05$, Figure 3M), total catecholamine (+40% *vs* C, $p < 0.05$, Figure 3N). NIC females showed higher protein content of ACC (+43% *vs* C, $p < 0.05$, Fig. 4I), CEBPβ (+41% *vs* C, $p < 0.05$, Fig. 4I), while no change was observed in β3-AR or total catecholamine (Figures 4M and N).

3.4. Hypothalamic MC4R and AMPK expressions

At PN120, NIC animals of both sexes showed no alteration in protein content of MC4R in the PVN and pAMPK/AMPK ratio in the VMH (Fig. 5A, B, E and F). At PN180, NIC males showed higher pAMPK/AMPK ratio in the VMH (+60% *vs* C, $p < 0.05$, Fig. 5D), while NIC females had no alteration in this parameter (Fig. 5H). No change of MC4R protein expression was detected in NIC animals (Fig. 5C and G).

4. Discussion

The reduction of the thermogenic capacity of the BAT is being discussed currently as a possible determinant for the development of obesity (Soler-Vázquez et al., 2018; Luijten et al., 2019a,b). We have previously reported that smoke exposure during lactation reduces, in the long-term, BAT SNS activity as well as thermogenesis (Peixoto et al., 2018). However, although a cigarette has more than 7,000 different substances (WHO, 2019; Rodgman and Perfetti, 2013), here we demonstrated that nicotine-only exposure during the breastfeeding period impairs thermogenesis, reducing sympathetic activity in the BAT in the adult offspring of both sexes, possibly inducing lower energy expenditure and constituting the main cigarette compound that induces those changes.

NIC males exhibited overweight, visceral fat accumulation and increased epididymal adipocyte area (de Oliveira et al., 2010), which is in agreement with present findings. NIC females had normal body mass and body fat, which are in accordance with a previous report (Pinheiro et al., 2011). Here, we further showed a higher WAT adipocyte area that, at least in part, could be associated with BAT hypofunction (lower SNS and thermogenesis markers). It is relevant to stress that the study of nicotine-programmed offspring at two different ages is important to show that the some disturbances at the molecular level are already present at PN120, such as reduced BAT thermogenic function and changes in adipogenesis and lipogenesis markers in the WAT,

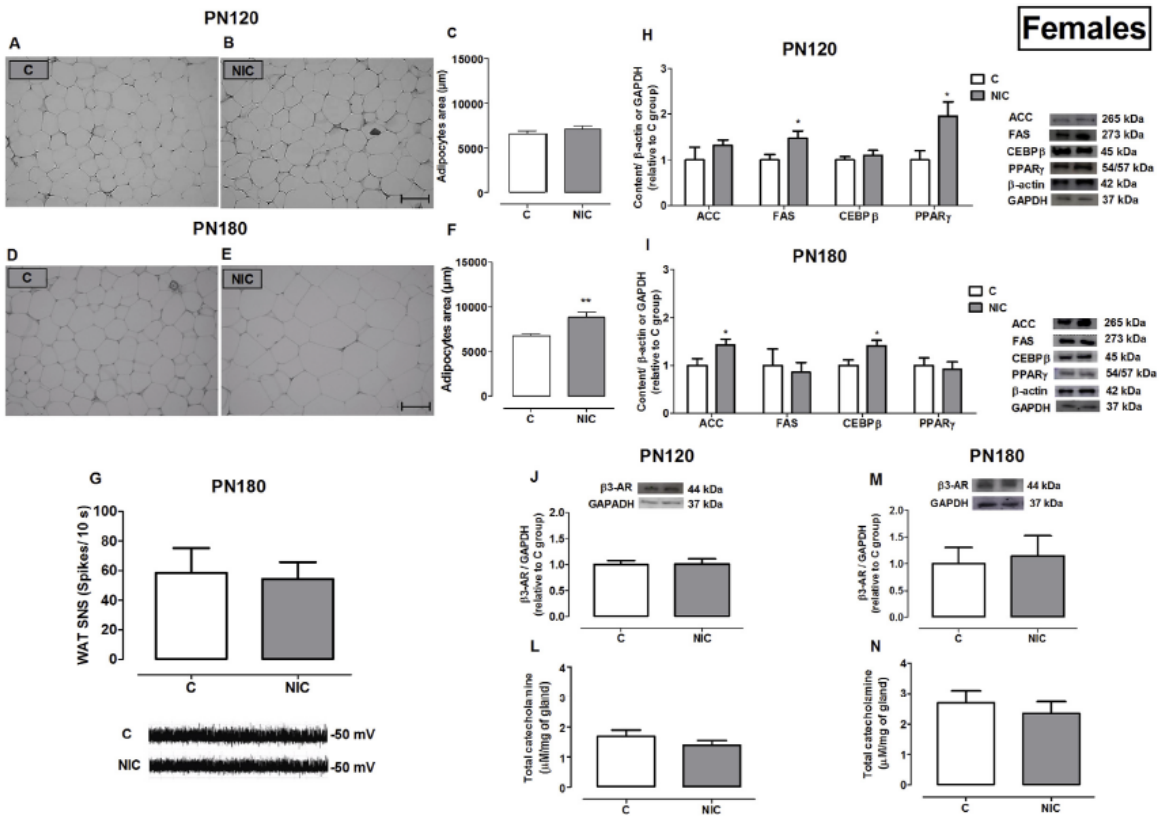

Fig. 4. Effect of nicotine-only exposure during breastfeeding on WAT in female offspring. Morphology and adipocytes area at PN120 (**A, B and C**) and PN180 (**D, E and F**) rat offspring. WAT SNS at PN180 (G). Lipogenic and adipogenic biomarkers (ACC, FAS, CEBPβ and PPARγ) at PN120 (**H**) and N180 (**I**). β3-AR protein contents at PN120 (**J**) and PN180 (**M**). Adrenal total catecholamine content at PN120 (**L**) and PN180 (**N**). **C**: control group; **NIC**: nicotine group. Results are expressed as mean ± SEM; *p < 0.05; **p < 0.01, n = 7.

observations that precede the morphological changes. These alterations intensify with aging and culminate in the morphological changes observed in the BAT and WAT at PN180.

In obesity, there is a continuous state of positive energy balance, hypertrophy and hyperplasia, increasing lipid storage (Rutkowski et al., 2015; Sun et al., 2011). Here, we demonstrated that despite the increased adipocyte area in the WAT, NIC males at PN180 showed increased WAT SNS, potentiating lipolysis. Besides, adipocyte hypertrophy results in increased basal lipolysis, favoring the release of FFA and lipid accumulation at ectopic sites (Lambert et al., 2019). Additionally, the increase, in NIC offspring, of adipogenic and lipogenic markers at PN120 (PPARγ and FAS in both males and females) and at PN180 (PPARγ and FAS in males; CEBPβ and ACC in females) also help to explain the morphological alteration in the WAT (Hammarstedt et al., 2018; Song et al., 2018). Studies show that SNS-BAT connection is indispensable for thermogenesis (Labbe et al., 2015; Morrison et al., 2014). Our findings demonstrate that BAT hypofunction is related to impaired SNS activity in NIC animals, irrespective of sex, at PN120 and PN180.

At PN180, NIC offspring of both sexes showed increased percentage of BAT lipid droplet area. Thus, NIC males developed BAT hypertrophy and hyperplasia, while females only had hypertrophy. Accordingly, as already reported, BAT whitening leads to cell death and tissue inflammation, impairing its function (Kotzbeck et al., 2018). Indeed, Fan and collaborators (2018) have reported that nicotine exposure during gestation and lactation alters BAT morphology and function in the adult male progeny.

In the present study, at both ages, NIC animals showed decreased UCP1 and CPT1a protein expressions, while other thermogenic markers showed sex-dependent alterations at PN120 and/or PN180. Fan et al. demonstrated that nicotine exposure during gestation and lactation reduces the expression of UCP1 and PGC1α mRNA as well as of other thermogenesis-related biomarkers (PRDM16, CIDEA, and CPT2). We evidenced, in the present study, that nicotine-only exposure exclusively during the breastfeeding period can also impair BAT thermogenesis biomarkers at adulthood in both sexes. Although BAT function depends on many different factors, such as temperature, food intake, physical activity, metabolic status and hormone action, its thermogenic capacity is proportional to the amount of UCP1, considered a key molecule regarding facultative thermogenesis (Jung et al., 2019; Kalinovich et al., 2017; Oelkrug et al., 2015). Thus, the reduction in the protein content of UCP1 in NIC animals at both ages is closely related to the reduction of thermogenic capacity observed in these animals. Furthermore, the observed reductions in other BAT biomarkers, such as β3-AR, CPT1a and PGC1α, could be due to the decreased SNS activity.

Thyroid status is related to the regulation of thermogenesis since the thyroid hormone exhibit a crosstalk with β3-AR (primarily responsible for UCP1 activation) (Phillips, 2019). Thyroid hormone receptors (TR) have specific actions in the regulation of thermogenesis; TRβ stimulates UCP1, while TRα enhances β-adrenergic signaling (Ribeiro et al., 2001;

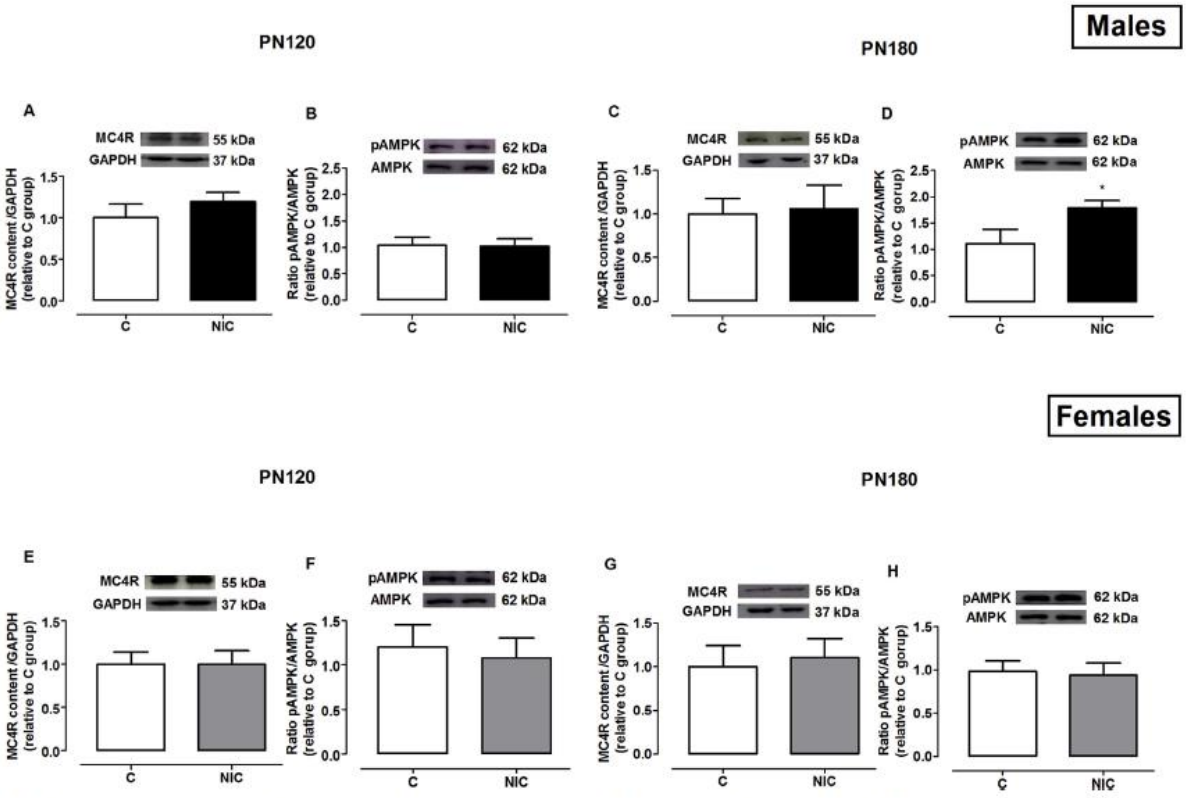

Fig. 5. Long-term effects of nicotine-only exposure during breastfeeding on the hypothalamic nuclei (PVN and VMH). MC4R protein contents in PVN at PN120 in male (**A**) and female (**E**). Ratio pAMPK - AMPK in VMH at PN120 in male (**B**) and female (**F**) rat offspring. MC4R protein contents in PVN at PN180 in males (**C**) and females (**G**). pAMPK/AMPK ratio in VMH at PN180 in males (**D**) and females (**H**). **C**: control group; **NIC**: nicotine group. Results are expressed as mean ± SEM; *p < 0.05; **p < 0.01, n = 7.

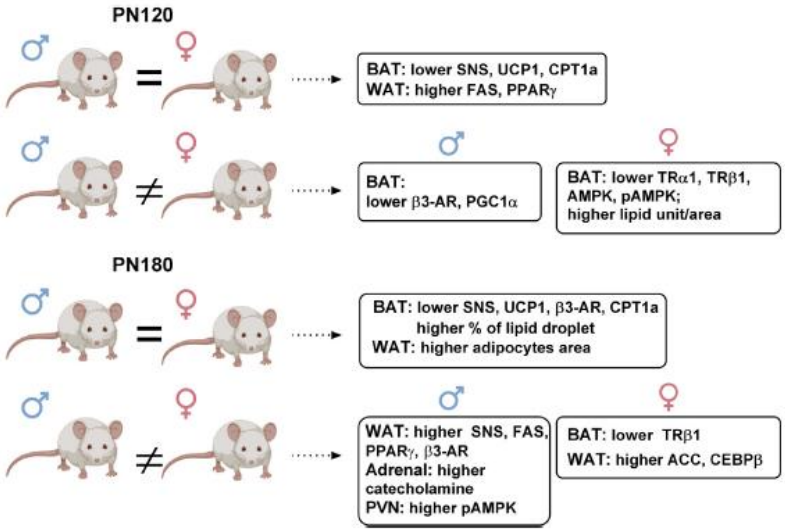

Fig. 6. Summary of key findings about sex-related differences in adult offspring in response to nicotine-only exposure during breastfeeding.

Martínez-Sánchez et al., 2014). Oliveira and collaborators (2009) demonstrated that NIC males show secondary hypothyroidism (lower circulating TSH, T3 and T4 concentrations) at PN180. Thus, the lower thermogenesis observed in this group may be related to its hypothyroid status. We did not measure thyroid hormones in the female offspring, which can be considered a limitation of the current study. However, NIC females had a reduction of TRα (both ages) and TRβ (only at PN120), which suggests a lower action of thyroid hormones in the BAT.

Central regulation of SNS-BAT involves the melanocortin system through the activation of MC4R in the PVN (Kooijman et al., 2014). The reduction of *in vivo* BAT SNS is not apparently dependent on this system in NIC animals. Another crucial hypothalamic marker for BAT thermogenesis is the AMPK located in the VMH (Wang and Cheng, 2018). AMPK content has an inverse relationship to thermogenesis in the BAT (López et al., 2016; Wang and Cheng, 2018). Thus, the increased pAMPK-AMPK ratio observed in the VMH of NIC males at PN180 seem to contribute to the lower *in vivo* SNS activity.

Here we evidence that there are sex-related differences in response to neonatal exposure to nicotine (Fig. 6). Some data show that women are more vulnerable to the effects of nicotine than men (Pogun and Yaratbas, 2009). Sex steroid hormones play an important role concerning the effects of nicotine in women: While estrogen increases the desire to smoke, progesterone decreases it (Lynch and Sofuoglu, 2010). It is important to note that the effects of nicotine vary according to developmental stage (prenatal, neonatal, adolescence and adults), existing strong evidence that male and female animals are differently programmed by nicotine (Cross et al., 2017). Previous and current data in this model indicate that male offspring is more susceptible to neonatal nicotine exposure. NIC male offspring developed overweight, increased visceral fat mass, adipocyte hypertrophy and higher leptin concentrations. In addition, specifically in the present work, we report greater BAT mass, greater WAT sympathetic activity and greater catecholamine. NIC female offspring were also programmed for an unfavorable phenotype, but changes were mild when compared to males.

5. Conclusions

BAT hypofunction has been associated with obesogenesis while thermogenesis activation by brown adipocytes has been associated with body mass and fat reductions, better glucose and insulin sensitivity and better lipid profile. This is the main reason why the control of thermogenesis has been viewed as an anti-obesity therapeutic strategy (Phillips, 2019; Soler-Vázquez et al., 2018; Stanford et al., 2013). Considering that obesity is a public health problem and is a potential condition for the development of other diseases, our current findings provide evidence of how neonatal nicotine exposure affects the SNS, metabolic biomarkers and morphology in the WAT and BAT in the long-term, considerably after the exposure to nicotine was interrupted. This knowledge contributes to the understanding that nicotine is the main substance underlying the mechanisms by which maternal smoking affects thermogenesis, resulting in obesity later in life.

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CRediT authorship contribution statement

T.C. Peixoto: Conceptualization. **E.G. Moura:** Conceptualization. **P.N. Soares:** Methodology, Investigation. **I.M. Bertasso:** Methodology, Investigation. **C.B. Pietrobon:** Methodology, Investigation. **F.A.H. Caramez:** Methodology, Investigation. **R.A. Miranda:** Methodology, Investigation. **E. Oliveira:** Methodology, Validation, Investigation, Resources, Funding acquisition. **A.C. Manhães:** Methodology, Validation, Formal analysis, Visualization. **P.C. Lisboa:** Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.4 **Artigo 4**: **Early Weaning Alters the Thermogenic Capacity of Brown Adipose Tissue in Adult Male and Female Rats (artigo publicado)**

T C Peixoto, C B Pietrobon, I M Bertasso, F A H Caramez, C Calvino, T R Santos, E Oliveira, E G Moura, P C Lisboa

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ORIGINAL CONTRIBUTION

Early weaning alters the thermogenic capacity of brown adipose tissue in adult male and female rats

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Abstract

Purpose Early weaning (EW) is a risk factor for obesity development. Brown adipose tissue (BAT) hypofunction is related to obesity onset. Here, we evaluated whether sympathetic nervous system (SNS) activity in BAT and the thermogenic function of BAT are decreased in adulthood in obese rats from two EW models.

Methods At the time of birth, lactating Wistar rats and their pups (three males and three females) were separated into three groups: the control group, in which pups consumed milk throughout lactation; the non-pharmacological EW (NPEW) group, in which suckling was interrupted with a bandage during the last 3 days of lactation; and the pharmacological EW (PEW) group, in which dams were treated with bromocriptine (0.5 mg/twice a day) 3 days before weaning. The offspring were sacrificed on PN180.

Results Adult male rats from both EW models exhibited lower BAT SNS activity. Female rats from the PEW group showed a decrease in BAT SNS activity. The protein levels of UCP1 were lower in the NPEW males, while PGC1α levels were lower in both PEW and NPEW males. Both groups of EW females showed reductions in the levels of β3-AR, TRβ1, and PGC1α. The UCP1 protein level was reduced only in the NPEW females. The EW groups of both sexes had lower AMPK protein levels in BAT. In the hypothalamus, only the PEW females showed an increase in AMPK protein levels. In both groups of EW males, adrenal catecholamine was increased and tyrosine hydroxylase was decreased, while in EW females, adrenal catecholamine was decreased.

Conclusions Early weaning alters the thermogenic capacity of BAT, which partially contributes to obesity in adulthood, and there are sex-related differences in these alterations.

Keywords Breastfeeding · Early weaning · Bromocriptine · Developmental plasticity · Metabolic syndrome · Autonomic function

Introduction

Environmental and nutritional changes during periods of great plasticity, such as intrauterine life and/or lactation, can cause metabolic disorders in offspring. This phenomenon is known as metabolic programming [1–4]. Early weaning can lead to metabolic programming. Epidemiological data have shown that exclusive breastfeeding up to 6 months of age is protective against the development of obesity in adulthood [5–8]. In rats, we have shown the long-term adverse effects of breast milk deprivation, which include programming for metabolic syndrome [9–12]. In a pharmacological early weaning (PEW) model, bromocriptine, a type 2 dopamine agonist that inhibits prolactin production/secretion, is administered to mothers during the last 3 days of lactation, and this programs adult male offspring for obesity, hyperleptinemia, insulin resistance, dyslipidaemia, central hypothyroidism, and high concentrations of adrenal hormones [9, 10]. Lima et al. [11, 12] developed a non-pharmacological early weaning (NPEW) model in which a bandage is applied to mothers during the last 3 days of lactation. They found that adult males are programmed for obesity, higher body fat, insulin resistance, hyperleptinemia, and hypertriglyceridemia [11, 12]. The aforementioned metabolic and hormonal alterations

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can be associated with changes in the function of brown adipose tissue (BAT), impairing its thermogenic capacity and favoring the development and maintenance of obesity [13]. Until now, female progeny have not been evaluated in these two EW models.

Brown adipocytes have a large number of mitochondria rich in uncoupling protein 1 (UCP1) [14]. UCP1 is a protein capable of decoupling the mechanism of oxidative phosphorylation from the respiratory chain, producing heat, and thereby increasing thermogenesis [15, 16]. The sympathetic nervous system (SNS) and thyroid hormones are responsible for regulating thermogenesis; therefore, beta 3-adrenergic receptor (β3-AR) and thyroid hormone receptors (TRβ1 and TRα1) [17] are considered important biomarkers of BAT function. There are other molecules involved in the control of thermogenesis, such as peroxisome proliferator-activated receptor coactivator (PGC1α), which regulates mitochondrial biogenesis and respiratory function, and carnitine palmitoyltransferase 1A (CPT1a) [18], which is involved in the control of mitochondrial fatty acid oxidation [19].

The hypothalamus has an important role in the regulation of BAT thermogenesis. The ventromedial nucleus of the hypothalamus (VMH), through AMP-activated protein kinase (AMPK), inhibits SNS activity [20, 21]. Another central regulator of BAT thermogenesis is the melanocortin system [22–24]. Alpha-melanocyte-stimulating hormone (α-MSH) binds to melanocortin 4 receptor (MC4R) in the paraventricular nucleus (PVN), thereby increasing BAT sympathetic outflow, stimulating mitochondrial biogenesis, and UCP1 production, and consequently increasing thermogenesis [22–24].

Studies have shown that increased BAT thermogenesis represents a potential mechanism against obesity [16, 25]. Thus, our hypothesis is that early weaning can act as an imprinting factor that results in the failure of BAT thermogenic function, contributing to obesogenesis. Here, we studied the long-term effects of early weaning in two models on: (1) BAT sympathetic nerve activity; (2) BAT morphology; (3) biomarkers of catecholamine and thyroid hormone sensitivity, mitochondrial biogenesis, and fatty acid oxidation in BAT; (4) total catecholamine levels in the adrenal gland; and (5) AMPK levels in BAT and the VMH as well as MC4R levels in the PVN. In addition, the sexual dimorphism of outcomes in adult progeny was also investigated.

Materials and methods

The Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based its analysis on the principles adopted and promulgated by Brazilian Law (No. 11.794/2008), approved our experimental design (CEUA/035/2017).

Wistar rats were housed under controlled temperature (23/24 °C) and a photoperiod of 12 h (light/dark cycle) with water and food ad libitum. Virgin adult female rats were placed with male rats at a 2 to 1 ratio for 1 week. After mating, each pregnant rat was housed in an individual cage until delivery. At the time of birth, which was considered the first post-natal day (PN1), the litter of each dam was adjusted to six pups (three females and three males).

Experimental models of early weaning (EW)

After birth, 21 lactating rats were randomly separated into 3 groups: the control (C, $n=7$) group, in which the pups experienced standard lactation (21 days); the non-pharmacological early weaning (NPEW, $n=7$) group, in which dams were wrapped with a bandage to interrupt lactation in the last 3 days of lactation; and the pharmacological early weaning (PEW, $n=7$) group, in which dams were treated with 0.5 mg bromocriptine i.p. (BRO; Novartis, São Paulo, Brazil) twice a day for 3 days at the end of lactation. According to Quinn (2005), one rat day is equivalent to 8.6 human days [26]. Thus, the standard breastfeeding period of a rat (21 days) corresponds to the recommended 6 months of exclusive breast feeding in humans. In this sense, 3 days of EW in rats are equivalent to almost 1 month of EW in humans.

For all groups, food was administered directly into the cages, and the pups had easy access to a water bottle. We used two randomly chosen pups (one male and one female) from each dam. The remaining pups were used for another study.

Between weaning (PN21) to euthanasia (PN180), the male and female pups were kept in different cages (three males and three females per cage). The estrous cycle was analyzed after PN150. The females were sacrificed during diestrous. All females had regular 4–5-day estrous cycles, indicating that programming induced by early weaning did not affect the reproductive cycle.

Sympathetic nervous system (SNS) activity in brown adipose tissue (BAT)

On PN180, one rat of each sex from each litter of all groups (C, NPEW and PEW) was fasted for 12 h and then anesthetized (70 mg/kg b.w. and 7 mg/kg b.w. xylazine) for in vivo autonomic nerve electrical activity assessment, as previously described [27]. BAT sympathetic nerve activity was measured from the left interscapular nerve, which was exposed under a dissection microscope. The branches were placed on a pair of hook platinum electrodes connected to an electronic device (Bio-Amplificator, Insight®, Ribeirão Preto, SP, Brazil) to record the electrical signals. To prevent dehydration, the nerve was covered with mineral oil. Nerve activity was amplified (10,000×) and filtered (cutoff:

60 kHz). The results were analyzed using the PowerLab data acquisition system (8SP; AD Instruments, New South Wales, Australia). All nerve activity recordings were carried out inside a Faraday cage to prevent electromagnetic interference. The animals were kept under a warming light. After 10 min of stabilization, the average number of spikes per 10-s interval during a 10-min period was calculated. The background noise level was determined in a nerve segment.

Euthanasia and tissue collection

After the measurement of BAT SNS activity, the rats were euthanized by cardiac puncture. The interscapular brown adipose tissue (iBAT) was dissected, weighed, and prepared for morphological studies or molecular measurements (stored at − 80 °C). The visceral fat compartments (mesenteric, gonadal, and retroperitoneal) were collected to obtain the total visceral fat mass (VFM). The whole brain was removed and stored at − 80 °C until the nuclei of interest were dissected.

BAT morphological analysis

Samples were fixed in paraformaldehyde (4%) and embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) to produce 5-µm thick non-serial sections. These sections were fixed onto glass slides (three per slide) for hematoxylin/eosin staining, and digital images were acquired randomly (TIFF format, 36-bit colour, 1360× 1024 pixels) using an Olympus DP71 camera and an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan). At least ten photomicrographs per animal were randomly measured with Image-Pro Plus 5.0 software (Media Cybernetics, Silver Spring, MD, USA). The photomicrographs were used for the selection of fat droplets. Digital images of the droplets were analyzed, the area of the droplets were determined, and the resulting data were transformed into a histogram.

Isolation of the hypothalamic nuclei

We used a cryostat (Hyrax C52, Zeiss, Germany) to obtain coronal sections of the brain. The paraventricular nucleus (PVN, − 0.6 to − 2.1 mm from bregma) and ventromedial nucleus of the hypothalamus (VMH, − 2.1 to − 3.6 mm from bregma) were isolated in accordance with the coordinates described in the Paxinos and Watson stereotaxic atlas [28]. The samples were frozen at − 80 °C for Western blotting.

Western blotting analysis

Western blotting was used to evaluate protein levels in BAT, the adrenal gland, the PVN, and the VMH. To homogenize the samples, we used RIPA buffer [50 mM Tris–HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF] with protease inhibitor cocktail (F. Hoffmann-La Roche Ltd., Basel, CH). A total of 700 µL of the buffer was used for BAT, 500 µL was used for the adrenal gland, 70 µL for PVN, and 70 µL VMH. The BAT was sonicated (three pulses of 5 s with 40% amplitude separated by 15 s); the adrenal gland was manually macerated using a laboratory pestle; and the PVN and VMH were sonicated (two pulses of 10 s with 40% amplitude separated by 15 s). The BAT homogenates were centrifuged three times (18.506×*g*, 5 min, 4 °C), and the intermediate phase was collected after each centrifugation. The protein concentration in the supernatants was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, CA, USA). Then the homogenates were analyzed by SDS-PAGE using 30 mg total protein for BAT and 10 mg total protein for the adrenal gland, PVN, and VMH. The samples were transferred onto PVDF membranes (Hybond ECL; Amersham Pharmacia Biotech, London, UK). The membranes were incubated with Tris-buffered saline (TBS) containing 5% albumin for 45 min. Subsequently, the membranes were washed with TBS and then incubated overnight at 4 °C with the following specific primary antibodies: anti-UCP1 (1:500; Sigma-Aldrich, Invitrogen Corporation CA, USA), anti-β3-AR (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-TRβ1, anti-TRα1 (1:500; Abcam, MA, UK), anti-PGC1α and CPT1a (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The same procedure was performed for the adrenal gland, VMH, and PVN membranes, which were incubated with anti-TH (tyrosine hydroxylase) (mouse; 1:1000; Sigma-Aldrich, Saint Louis MO, USA), anti-AMPKα, anti-phospho-AMPKα (1:500; Cell Signaling Technology, Inc., MA, USA), and anti-MC4R (1:500; Abcam, MA, UK) antibodies. An anti-β-actin primary antibody was used as an internal control for each membrane (1:500; Sigma-Aldrich, Invitrogen Corporation, CA, USA). The membranes were washed three times with Tween-TBS (0.1%) and then incubated for 1 h at room temperature with the appropriate concentration (1:1000, 1:5000, 1:7000 or 1:10000) of a secondary antibody conjugated to biotin (anti-rabbit, anti-mouse or anti-goat; Sigma-Aldrich, Invitrogen Corporation CA, USA). Then the membranes were washed three more times with Tween-TBS (0.1%) and then incubated with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA, USA). The protein bands were visualized with chemiluminescence (ECL Plus kit; Amersham Biosciences, London, UK) and exposed to ImageQuant LAS (GE Healthcare, Buckinghamshire, UK). The area and density of the bands were quantified by ImageJ software (Wayne Rasband National Institute of Health, MA, USA) and normalized against the bands obtained for β-actin. The results are expressed as the percent change relative to the control group (C).

Determination of the total catecholamine level in the adrenal gland

The total catecholamine level was measured using the trihydroxyindole method [29]. The right adrenal gland was used. The adrenal glands were mashed in 10% acetic acid until a homogenate was obtained and the homogenate was then centrifuged (1120×*g*, 5 min, 4 °C). The supernatants were frozen for analysis. Epinephrine was used as a standard. Briefly, 50 µl of epinephrine or the supernatant was mixed with 250 µL of 0.5 M phosphate buffer, pH 7.0, and 25 µL of 0.5% potassium ferricyanate and incubated (20 min, ice bath). The reaction was stopped with 500 µL of 60 mg/mL ascorbic acid and 5 N NaOH (1:19 ratio), and the samples were diluted with 2 mL of distilled water. The fluorescence was determined at 420 nm for excitation and 510 nm for emission (Hidex, Turku, FI).

Statistical analysis

The data were analyzed with the statistical program GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA) and are expressed as the means ± standard error of the mean (SEM). Each variable was analyzed by two-way ANOVA with programming and sex as between-subjects factors, as depicted in Table 1. If the initial analysis indicated effects of both programming and sex or an interaction between these factors, the data were re-examined by one-way ANOVA followed by the Newman–Keuls post hoc test. Differences were considered significant at $p < 0.05$.

Results

In vivo BAT SNS activity

For BAT SNS activity, we observed an effect of programming ($F_{2,31} = 9.17$) and an interaction between the factors (programming and sex, $F_{2,31} = 3.71$) but no effect of sex. In both EW models (Fig. 1a), males showed lower BAT SNS activity under basal conditions compared to that of the C group (NPEW, − 68% and PEW, − 37% vs C; $p < 0.05$). The NPEW males also exhibited lower BAT SNS activity compared to that of the PEW group (NPEW, − 51% vs PEW; $p < 0.05$; Fig. 1a). Only the PEW females showed lower BAT SNS activity (− 57%; $p < 0.05$; Fig. 1b) compared to that of the C group. We observed an interaction between the factors (programming and sex; $F_{2,31} = 3.71$), with the NPEW females exhibiting an increase in BAT SNS activity compared to that of NPEW males (+ 2.2-fold vs NPEW males; $p < 0.05$; Fig. 1).

Table 1 Effect of non-pharmacological and pharmacological early weaning on analyzed parameters in animals at PN180

	Programming effect	Sex effect	Interaction
Body weight (g)	Yes ($F = 7.94$, $p = 0.0012$)	Yes ($F = 660$, $p < 0.0001$)	No ($F = 3.1$, $p = 0.055$)
VFM (g)	Yes ($F = 16.85$, $p < 0.0001$)	Yes ($F = 70.23$, $p < 0.0001$)	Yes ($F = 5.84$, $p = 0.006$)
VFM (g)/100 g BW	Yes ($F = 17.93$, $p < 0.0001$)	NNo ($F = 0.42$, $p = 0.52$)	No ($F = 1.03$, $p = 0.368$)
iBAT mass (g)	No ($F = 0.17$, $p = 0.845$)	Yes ($F = 15.5$, P = 0.0004)	No ($F = 1.66$, $p = 0.205$)
iBAT mass (g)/100 g BW	No ($F = 0.7$, $p = 0.506$)	Yes ($F = 12.67$, $p = 0.0012$)	No ($F = 2$, $p = 0.152$)
BAT SNS	Yes ($F = 9.17$, $p = 0.0007$)	No ($F = 0.04$, $p = 0.841$)	Yes ($F = 3.71$, $p = 0.0358$)
Lipid droplets (% area)	No ($F = 1.68$, $p = 0.2034$)	No ($F = 2.56$, $p = 0.091$)	No ($F = 1.89$, $p = 0.1662$)
UCP1 content	Yes ($F = 8.10$, $p = 0.0016$)	No ($F = 0.29$, $p = 0.5971$)	No ($F = 1.25$, $p = 0.3018$)
β3-AR content	No ($F = 0.86$, $p = 0.4309$)	Yes ($F = 12.8$, $p = 0.0010$)	Yes ($F = 3.34$, $p = 0.0465$)
TRα1 content	No ($F = 2.44$, $p = 0.1019$)	Yes ($F = 11.09$, $p = 0.002$)	No ($F = 3.10$, $p = 0.0580$)
TRβ1 content	No ($F = 1.25$, $p = 0.2986$)	Yes ($F = 14.35$, $p = 0.0005$)	Yes ($F = 3.82$, $p = 0.0306$)
PGC1α content	Yes ($F = 15.71$, $p < 0.0001$)	No ($F = 0.01$, $p = 0.9788$)	No ($F = 0.02$, $p = 0.9419$)
CPT1a content	No ($F = 1.21$, $p = 0.3138$)	No ($F = 0.05$, $p = 0.194$)	No ($F = 0.05$, $p = 0.8231$)
BAT- ratio AMPKp/AMPK	Yes ($F = 17.23$, $p < 0.0001$)	No ($F = 2.8$, $p = 0.1030$)	No ($F = 0.78$, $p = 0.4662$)
µg catecholamine/mg tissue	No ($F = 0.83$, $p = 0.4407$)	Yes ($F = 29.9.12$, $p < 0.0001$)	Yes ($F = 13.96$, $p < 0.0001$)
TH content	Yes ($F = 3.82$, $p = 0.029$)	Yes ($F = 13.44$, $p = 0.0007$)	Yes ($F = 3.58$, $p = 0.0367$)
VMH-ratio AMPKp/AMPK	No ($F = 2.5$, $p = 0.0934$)	Yes ($F = 9.82$, $p = 0.003$)	Yes ($F = 3.4$, $p = 0.042$)
MC4R content	No ($F = 1.11$, $p = 0.34$)	No ($F = 1.48$, $p = 0.232$)	No ($F = 0.37$, $p = 0.69$)

VFM visceral fat mass, *BW* body weight, *iBAT* interscapular brown adipose tissue, *UCP1* uncoupling protein 1, *β3-AR* beta 3-adrenergic receptor, *TRα1* thyroid hormones receptor alpha 1, *TRβ1* thyroid hormones receptor beta 1, *PGC1α* peroxisome proliferator-activated receptor coactivator, *CPT1a* carnitine palmitoyltransferase 1A, *AMPKp* AMP-activated protein kinase phosphorylated, *AMPK* AMP-activated protein kinase, *TH* tyrosine hydroxylase, *MC4R* melanocortin 4 receptor

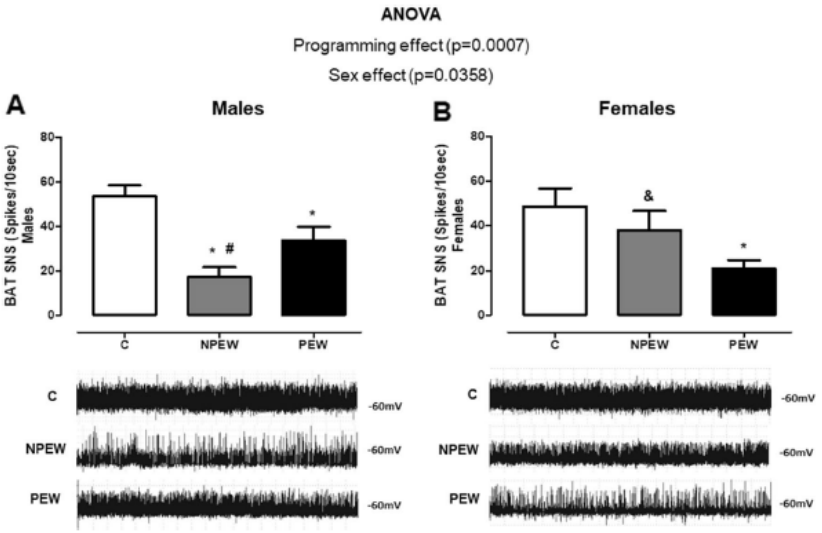

Fig. 1 Long-term effects of early weaning on in vivo BAT SNS. Number of spikes in 10 s at PN180 in males (**a**) and females (**b**). Representative recordings are shown at the bottom of the figures. *C* control group, *NPEW* non-pharmacological early weaning group, *PEW* pharmacological early weaning group. Results are expressed as mean ± SEM. $^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\phi}p<0.05$ vs. PEW male, $n=7$

Fat deposits

As shown in Table 2, there was an effect of programming ($F_{2,40}=7.94$) on body weight, with the EW groups having higher body weights than those of the respective C groups (males: NPEW, +9.4% and PEW, +13.4%; females: NPEW, +8.7% and PEW +8%; p < 0.05). An effect of sex ($F_{1,40}=660$) on body weight was observed, with a clear difference between males and females.

For visceral fat mass, we observed effects of programming ($F_{2,41}=16.85$) and sex ($F_{1,41}=70.23$) and an interaction between the factors ($F_{2,41}=5.84$). The males from the NPEW and PEW groups had higher visceral fat mass compared to that of the C group (NPEW, +60% and PEW +90%; $p<0.05$; Table 2). To a lesser degree, the females from both EW groups exhibited more visceral fat deposits than the C group (NPEW, +21% and PEW, +34%; $p<0.05$; Table 2).

There was an effect of programming only ($F_{2,34}=17.93$) on the fat and body weight ratio, with the EW groups having a higher VFM than that of the respective C groups (males: NPEW, +39.4% and PEW +54.4%; females: NPEW, +15% and PEW, +42.6%; $p<0.05$; Table 2).

For iBAT mass and iBAT mass/BW, we observed an effect of sex (iBAT, $F_{1,33}=15.584$ and iBAT mass/BW, $F_{1,21}=12.67$). The PEW females had a lower iBAT mass compared to that of PEW males (−68.7%), and the NPEW group showed an increase in iBAT mass/BW compared to that of NPEW males (+59%; $p<0.05$; Table 2).

BAT morphology and thermogenic biomarkers

As depicted in Fig. 2, there was no significant difference between the lipid droplet section area among the groups in both male and female offspring.

Table 2 Long-term effect of early weaning on body weight, fat mass, and iBAT mass of the offspring

	MALES			FEMALES		
	C	NPEW	PEW	C	NPEW	PEW
Body weight (g)$^{(P)(S)}$	425 ± 6.03	465 ± 6.64*	482.8 ± 14.6*	252.3 ± 1.43	274.2 ± 3.54$^{*\&}$	272.6 ± 2.45$^{*\phi}$
VFM (g)$^{(P)(S)(P+S)}$	13.86 ± 0.28	22.25 ± 1.5*	26.34 ± 4*	9.69 ± 0.39	11.72 ± 0.47$^{*\&}$	12.96 ± 0.71$^{*\phi}$
VFM (g)/100 g BW$^{(P)}$	3.44 ± 0.077	4.796 ± 0.19*	5.312 ± 0.36*	3.66 ± 0.10	4.21 ± 0.07*	5.22 ± 0.12*
iBAT mass (g)$^{(S)}$	0.34 ± 0.02	0.30 ± 0.05	0.32 ± 0.04	0.20 ± 0.01	0.26 ± 0.2	0.22 ± 0.01$^{\phi}$
iBAT mass (g)/100 g BW$^{(S)}$	0.0708 ± 0.0075	0.066 ± 0.011	0.075 ± 0.0087	0.083 ± 0.0045	0.1052 ± 0.006$^{\&}$	0.088 ± 0.0053

Values represent means ± SEM, $n=7$/group. $^{(P)}$ programming effect, $^{(S)}$ sex effect, $^{(P+S)}$ interaction.$^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\phi}p<0.05$ vs. PEW male

C control, *NPEW* non-pharmacological early weaning, *PEW* pharmacological early weaning, *VFM* visceral fat mass, *BW* body weight, *iBAT* interscapular brown adipose tissue

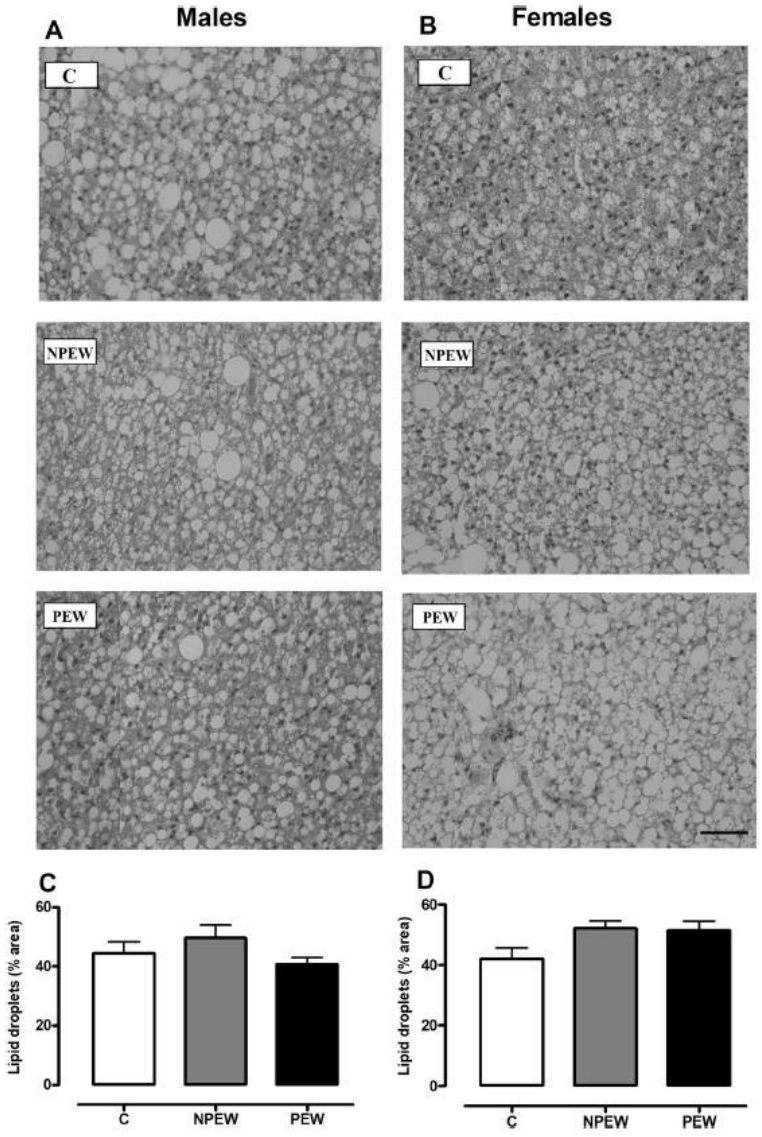

Fig. 2 Long-term effects of early weaning on BAT morphology. Representative hematoxylin and eosin staining of BAT at PN180 in males (**a**) and females (**b**). Percentage of brown adipocyte area in males (**c**) and females (**d**). *C* control group, *NPEW* non-pharmacological early weaning group, *PEW* pharmacological early weaning group. Results are expressed as mean ± SEM. $^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\Phi}p<0.05$ vs. PEW male, $n=7$

Concerning the protein content of thermogenesis biomarkers, there was a programming effect on UCP1 and PGC1α levels (UCP1, $F_{2,28}=8.10$ and PGC1α, $F_{2,30}=15.71$), a sex effect (β3-AR, $F_{1,37}=12.8$ and TRβ1, $F_{1,39}=14.35$) and an interaction between programming and sex (β3-AR, $F_{2,37}=3.34$ and TRβ1, $F_{2,39}=3.82$) on β3-AR and TRβ1 levels, an effect of sex on TRα1 levels ($F_{2,34}=11.09$), and no effect on CPT1a levels.

On PND180, the males from the NPEW group presented a reduction in the protein content of UCP1 (− 64% vs. C and − 64% vs. PEW) and PGC1α (− 49% vs. C) but no alterations in other BAT biomarkers (Fig. 3). The PEW males exhibited a reduction in the protein content of PGC1α compared to that in the C group (− 34%) but no change in other BAT biomarkers.

On PND180, NPEW females showed lower UCP1 protein levels (− 35% vs. C females; Fig. 3). Both female EW groups showed lower protein levels of β3-AR (NPEW, − 48% and PEW, − 36% vs. C females; − 58% vs. NPEW males and − 56% vs. NPEW males; $p<0.05$), TRβ1 (NPEW, − 40%

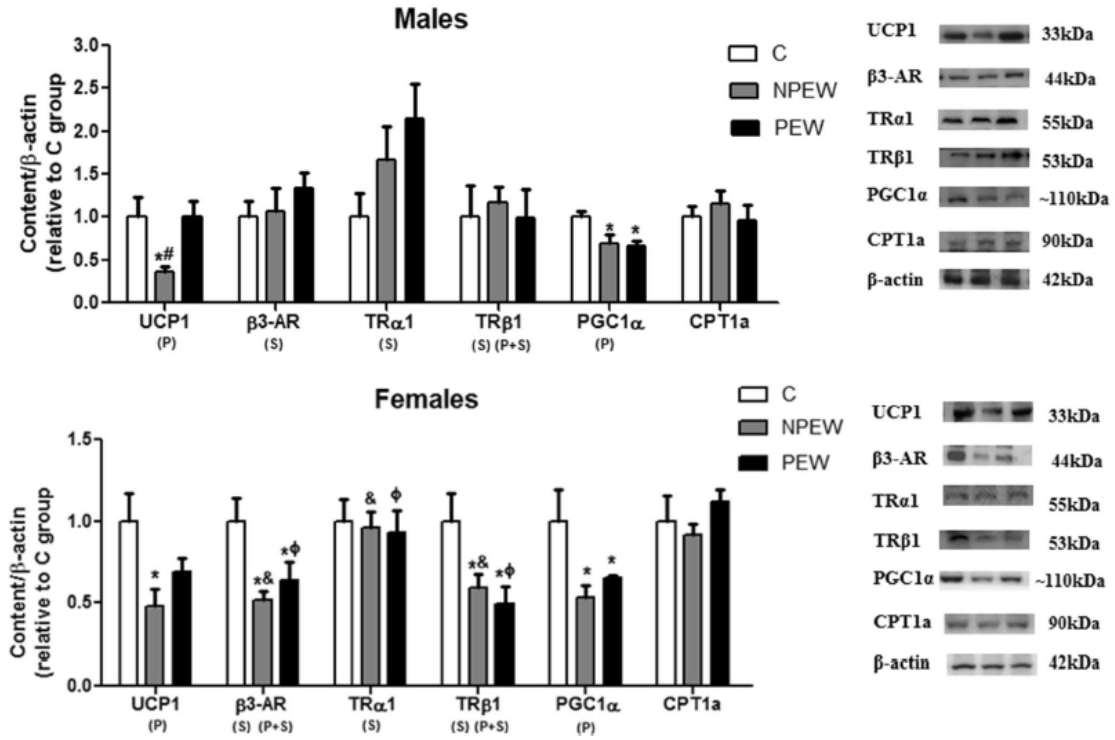

Fig. 3 Long-term effects of early weaning on BAT functional parameters in male and female offspring. UCP1, β3-AR, TRα1, TRβ1, PGC1α, and CPT1a protein contents in BAT at PN180 in males and females. Representative blots of the proteins are shown beside of the graphs. β-Actin content was used as control loading. *C* control group, *NPEW* non-pharmacological early weaning group; PEW: pharmacological early weaning group. Results are expressed as mean ± SEM. $^{(P)}$programming effect, $^{(S)}$ sex effect, $^{(P+S)}$ interaction. $^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\phi}p<0.05$ vs. PEW male, $n=6$–7

and PEW, − 51% vs. C females; $p<0.05$; − 57% vs. NPEW males and − 69% vs. NPEW males; $p<0.05$), and PGC1α (NPEW, − 46% and PEW, − 35% vs. C females; $p<0.05$) but no change in CPT1 levels (Fig. 3). The level of TRα1 was only lower when compared to that of males (− 42.7% vs. NPEW males; − 56% vs. PEW; $p<0.05$).

As depicted in Fig. 4, there was an effect of programming on the pAMPK/AMPK ratio ($F_{2,36}=17.23$), with the EW groups of both sexes exhibiting lower protein levels in BAT (males: NPEW, − 73% and PEW, − 69% vs. C; females: NPEW, − 44% and PEW, − 45% vs. C; $p<0.05$).

Adrenal medulla evaluation

There was an effect of sex ($F_{1,49}=29.9$) and an interaction between programming and sex ($F_{2,49}=13.96$) on catecholamine levels, whereas there were effects of programming ($F_{2,42}=3.82$) and sex ($F_{1,42}=13.44$) and an interaction between the factors ($F_{2,42}=3.58$) on TH levels. In both EW models, males showed higher total catecholamine levels (NPEW, + 2.3-fold and PEW, + 71% vs. C; $p<0.05$; Fig. 5a). On the other hand, EW females showed lower adrenal catecholamine levels (NPEW, − 33% and PEW, − 25% vs. C; $p<0.05$; Fig. 5c).

As depicted in Fig. 5b, both EW male groups presented lower TH protein levels (NPEW, − 56% and PEW, − 33% vs. C; $p<0.05$), whereas the females showed higher levels only when compared to those of males (+ 1.23-fold vs. NPEW males; + 66% vs. PEW males; Fig. 5d).

Hypothalamic modulation of BAT thermogenesis

There was an effect of sex ($F_{1,45}=9.82$) and an interaction between programming and sex ($F_{2,45}=3.4$) on the pAMPK/AMPK ratio in the VMH. As shown in Fig. 6a and b, there was no difference in the protein levels of MC4R in the PVN or the pAMPK/AMPK ratio in the VMH among the male groups. The PEW females showed an increased pAMPK/AMPK ratio in the VMH compared to that of the C group

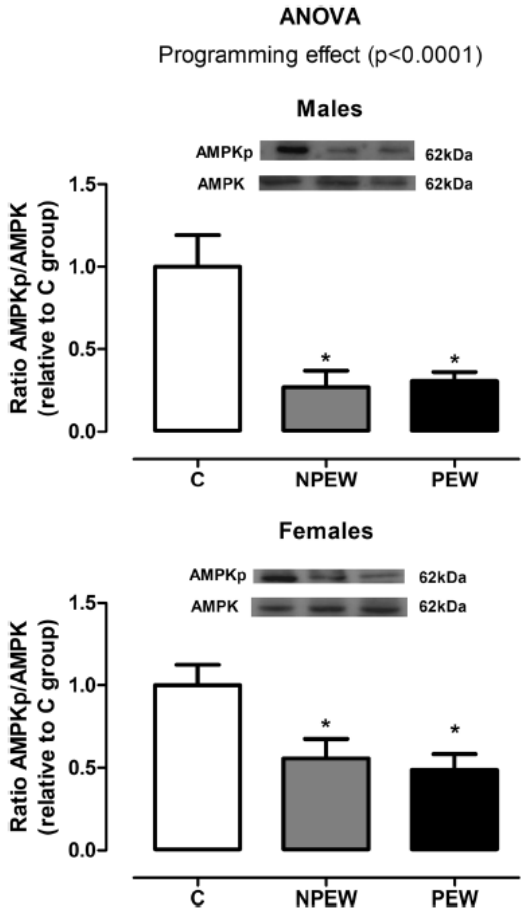

Fig. 4 Long-term effects of early weaning on the ratio of AMPKp/AMPK in the BAT. Ratio of AMPKp/AMPK in males (**a**) and females (**b**). Representative blots of proteins are shown above the graphs. Values are expressed as ratio of AMPKp and AMPK relative to the control group. *C* control group, *NPEW* non-pharmacological early weaning group, *PEW* pharmacological early weaning group. Results are expressed as mean ± SEM. $^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\Phi}p<0.05$ vs. PEW male, $n=6$–7

(+62.7%) and the NPEW group (+49.4%) but no change in the MC4R level in the PVN (Fig. 6c and d; $p<0.05$).

Discussion

BAT is an active endocrine organ in adults [30] and can be a target for the management of metabolic disorders such as obesity [31]. We found that early weaning programs the thermogenic capacity of BAT, either by reducing SNS activity or by altering important markers related to thermogenesis. Our findings are relevant to understanding the pathogenesis of obesity in EW models. BAT hypofunction may be partially dependent on decreased SNS activity in this tissue. Our data demonstrate that the reduction of in vivo BAT SNS activity in adulthood is not related to the melanocortin system in either EW males or females.

We showed that adult EW animals of both sexes have increased visceral adiposity, and the data from EW males corroborate that of former studies from Bonomo et al. [9] and Lima et al. [11, 12]. Although animals programmed by EW have higher visceral fat, the mechanisms by which SNS activity is reduced are different between the sexes and the two EW models. Despite the substantial changes in visceral adiposity, there was no change in BAT morphology in males or females from both EW models.

The males from both EW models presented lower in vivo BAT SNS activity compared to that of the C group, and the NPEW group exhibited BAT SNS activity that was even lower than that of the PEW group. Consequently, NPEW males have lower protein levels of UCP1 and PGC1α, while PEW males have only lower levels of PGC1α. Both UCP1 and PGC1α are quite important for normal BAT function and contribute to lower thermogenesis in these animals. However, in addition to the reduction in in vivo BAT SNS activity, we expected that the other markers would also be reduced. We did not find any change in the protein levels of β3-AR, TRα1, TRβ1, or CPT1a. One limitation of our study is that we did not evaluate the activity of these potentially reduced biomarkers in EW males because this tissue is less stimulated by SNS during EW and because these proteins are SNS dependent. Thus, we hypothesize that BAT hypofunction precedes tissue whitening. In fact, alterations in BAT function have already been reported without morphological changes [32].

Thyroid hormones are important regulators of BAT thermogenesis because TRβ1 increases the expression of UCP1 and TRα1 increases adrenergic activity [17]. Bonomo et al. [33] showed that adult PEW males present central hypothyroidism, which is characterized by a reduction in TRH, TSH, T4, and T3. Thus, at least in the PEW model, lower thermogenesis may be related to hypothyroidism. Additionally, the reduction in PGC1α in PEW males may be due to both hypothyroidism and lower adrenergic tonus in BAT [34]. Interestingly, NPEW males, despite their euthyroid status [12], exhibit decreased levels of both UCP1 and PGC1α exclusively due to the decrease in BAT SNS activity.

In males, other changes programmed by EW may be responsible for lower thermogenesis. Both NPEW and PEW males exhibit higher catecholamine levels and lower TH protein levels, possibly due to decreased epinephrine production associated with lower secretion or the inhibition of TH, the limiting enzyme for the production of catecholamines, by

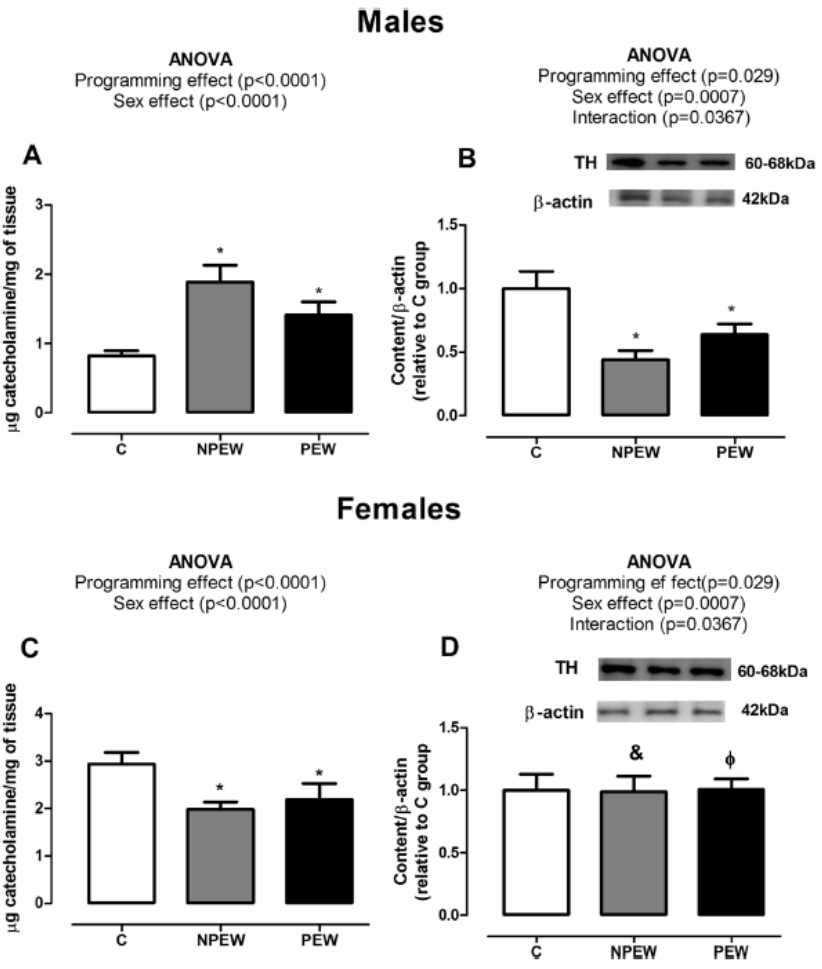

Fig. 5 Long-term effects of early weaning in the adrenal medulla. Total catecholamine content in males (**a**) and females (**c**). Protein content of TH in males (**b**) and females (**d**). Representative blots of proteins are shown above the graphs. β-Actin content was used as control loading. Values are expressed as relative (%) to the control group. *C* control group, *NPEW* non-pharmacological early weaning group, *PEW* pharmacological early weaning group. Results are expressed as mean ± SEM. $^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\phi}p<0.05$ vs. PEW male, $n=7$

the end product of biosynthesis (epinephrine). Therefore, it is possible that EW males have a lower adrenergic effect, even though β3-AR protein levels in BAT are not altered.

In females, the PEW group showed lower in vivo BAT SNS activity with a tendency for decreased UCP1 levels, while the NPEW group exhibited no change in in vivo BAT SNS activity and lower UCP1 levels. Thus, it is possible that the PEW females had lower UCP1 activity. Other factors, such as thyroid hormones and leptin [35], can regulate UCP1 independently of SNS activity. Another limitation of the current study is that we did not evaluate thyroid function or leptin in EW females.

Other BAT biomarkers, such as β3-AR, TRβ1, and PGC1α, which may contribute to reducing thermogenesis, are reduced in females from both EW models. The positive relationship between β3-AR and thermogenesis is well described in the literature, as UCP1 and PGC1α do not increase in mice without β-ARs in cold temperatures or at room temperature [36]. Despite no alteration in TH content, EW females from both models exhibited lower adrenal catecholamine levels, suggesting a reduction in the adrenergic load in BAT and a reduction in the thermogenic capacity of these animals. The TRβ1 reduction in EW females is another indicator of BAT hypofunction, which results in decreased thermogenesis. In the PEW females, the higher pAMPK/AMPK ratio in the VMH reinforces the reduction in in vivo BAT SNS activity since the activation of AMPK in the hypothalamus reduces energy expenditure and, consequently, thermogenesis in BAT [20].

We hypothesize that the reduction in the pAMPK/AMPK ratio in males and females from both EW models compromises the function of BAT and contributes to fibrogenesis and the dysfunction of adipocytes [37].

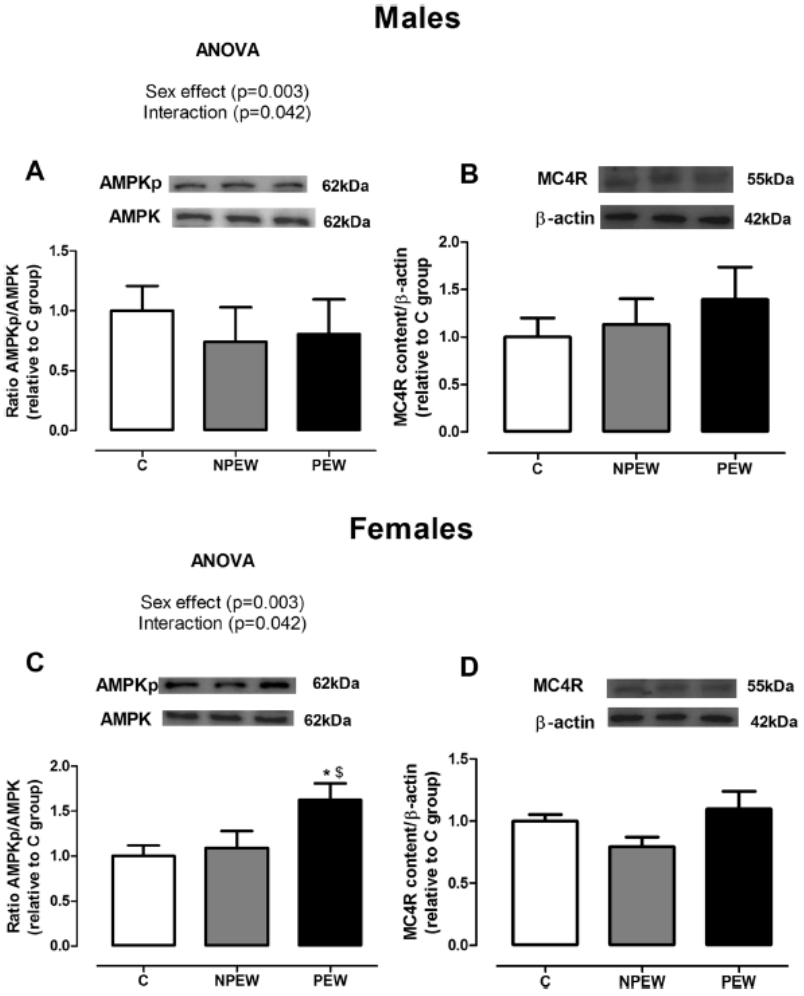

Fig. 6 Long-term effects of early weaning on ratio of AMPKp/AMPK in VMH and protein content MC4R in PVN. Ratio of AMPKp/AMPK in males (**a**) and females (**c**). Protein content of MC4R in males (**b**) and females (**d**). Representative blots of proteins are shown above the graphs. β-Actin content was used as control loading. Values are expressed as relative (%) to the control group. *C* control group, *NPEW* non-pharmacological early weaning group, *PEW* pharmacological early weaning group. Results are expressed as mean ± SEM. $^{*}p<0.05$ vs. C, $^{\#}p<0.05$ vs. PEW, $^{\$}p<0.05$ vs. NPEW (same-sex comparisons). $^{\&}p<0.05$ vs. NPEW male, $^{\Phi}p<0.05$ vs. PEW male, $n=7$

It is known that estrogen can reduce BAT thermogenesis [38–40], which may explain the different responses between the sexes found in the present study regarding thermogenic capacity. Studies have identified the proper functioning of BAT as a target of anti-obesity therapy and have indicated that BAT transplantation is capable of improving metabolic syndrome components and promoting improved glucose tolerance, increased insulin sensitivity, lower body weight, and decreased fat mass [41, 42]. In the literature, studies on thermogenesis and sex-related differences are scarce and still controversial [38–40]. Additionally, there is a scarcity of experimental studies on early weaning programming. To the best of our knowledge, our group is the first to study how experimental early weaning programs obesity and unravels the different mechanisms that lead to the development and maintenance of programmed obesity in both the sexes.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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4 **CONSIDERAÇÕES FINAIS**

Considerando o caráter pandêmico da obesidade e sua associação com diversas comorbidades, é importante identificar os distúrbios endócrino-metabólicos envolvidos na gênese da obesidade, fundamentando-se principalmente nos insultos que ocorrem em períodos de grande plasticidade. Dentre estes períodos, a maioria dos estudos concentra-se na gestação; no entanto, sabendo que o leite materno é a fonte primária de nutrição para o bebê e diversas substâncias podem ser transmitidas através do leite materno, nosso laboratório tem explorado o período de amamentação como uma importante janela crítica de programação, na qual exposições nutricionais (desmame precoce) e ambientais (componentes do cigarro), desencadeiam alterações metabólicas importantes.

No modelo de programação pelo tabagismo materno demonstramos os impactos negativos sobre a saúde em longo prazo, ressaltando a importância da interrupção do uso de cigarros ou produtos de nicotina por gestantes e lactantes. Desencorajamos ainda, o uso de adesivos de nicotina ou cigarros eletrônicos, que muitas vezes são utilizados como terapia para substituir o cigarro. A partir dos nossos resultados, observamos que a exposição precoce a fumaça do cigarro e/ou nicotina isolada, impacta diretamente no funcionamento do TAM e do hipotálamo da prole de ambos os sexos quando adultos.

Especificamente quanto ao modelo de programação pela exposição direta e indireta da prole à fumaça de cigarro durante a lactação sobre a função do TAM, os animais de ambos os sexos apresentam redução da atividade simpática do TAM e menor capacidade termogênica com alteração de importantes marcadores. No entanto, acreditamos que machos são mais afetados, uma vez que apresentam maior redução da atividade simpática e alterações de maior número de biomarcadores relacionados a termogênese. Nesse mesmo modelo, evidenciamos alterações hipotalâmicas que favorecem a hiperfagia e desenvolvimento de obesidade. As expressões de neuropeptídios hipotalâmicos são diferentes entre machos e fêmeas; enquanto machos apresentam aumento de NPY, CRH e redução de TRH, fêmeas apresentam redução de CART e α-MSH, embora tudo isto explique o fenótipo hiperfágico dos animas. Apenas os machos apresentam inflamação hipotalâmica; dados anteriores indicam que o principal componente do cigarro responsável pela inflamação central é a nicotina. Interessantemente, dados da literatura sugerem que, devido a presença de receptores de estrogênio e progesterona nos astrócitos, as mulheres apresentam uma maior proteção em relação ao desenvolvimento

de gliose hipotalâmica. Esta pode ser a base para as fêmeas do nosso modelo não terem apresentado alteração dos marcadores de inflamação no hipotálamo (GFAP, IL6, CX3CR1).

A nicotina é o principal composto bioativo do cigarro capaz de ser transferida pelo leite materno e, portanto, o modelo de exposição exclusiva a nicotina durante a amamentação traz importantes lições. Nossos achados demonstram os efeitos deletérios da exposição precoce à nicotina sobre o funcionamento do TAM e do TAB. Os animais expostos a nicotina de ambos os sexos, apresentam comprometimento da termogênese, com redução da atividade simpática e de marcadores, como UCP1. No TAB, apesar de apenas os machos apresentarem aumento de gordura visceral, a prole de ambos os sexos apresenta aumento da área dos adipócitos. Os machos apresentam ainda, aumento da atividade simpática no TAB, indicando maior lipólise. É possível que os ácidos graxos liberados nesse processo estejam sendo acumulados em outros tecidos (gordura ectópica). O conjunto dos dados anteriores e atuais neste modelo experimental indicam que a prole masculina é mais suscetível aos desfechos deletérios da exposição precoce à nicotina, apresentando um fenótipo mais desfavorável.

No modelo de programação pelo desmame precoce, abordamos a importância do aleitamento materno exclusivo, o qual é considerado uma estratégia eficaz e natural de vínculo, afeto, proteção e nutrição da mãe para o bebê. Resultados anteriores e atuais, sugerem que a interrupção precoce do aleitamento materno tem repercussões ao longo de toda a vida do indivíduo, impactando diretamente no desenvolvimento de obesidade e alterações metabólicas. Aqui demonstramos que o desmame precoce programa os filhotes para disfunção do TAM reduzindo atividade simpática em ambos os sexos.

Destacamos que estudos sobre termogênese e diferenças relacionadas ao sexo ainda são escassos e controversos. Os modelos de programação para obesidade abordados nesta Tese indicam que a redução da capacidade termogênica do TAM é um mecanismo importante para o desenvolvimento e manutenção da obesidade. Com os nossos resultados, esperamos contribuir e despertar novos interesses para o desenvolvimento de outras pesquisas, no que diz respeito a termogênese e inflamação hipotalâmica. Destacamos ainda, a necessidade de reforçar as ações já implementadas e desenvolver novas políticas de proteção, incentivo e apoio ao aleitamento materno, assim como de interrupção ao tabagismo não apenas no período gestacional, mas também durante a lactação, no intuito de minimizarmos a exposição a fatores de impressão, a fim de prevenir o surgimento de doenças crônicas na vida adulta como obesidade.

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ANEXO A - Aprovação do Comitê de Ética Modelo experimental fumaça

UNIVERSIDADE DO ESTADO DO RIO DE JANEIRO

INSTITUTO DE BIOLOGIA ROBERTO ALCANTARA GOMES

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS

C E R T I F I C A D O

Certificamos que o Protocolo nº **CEUA/019/2014** sobre **"Avaliação das alterações neurais, endócrinas e metabólicas no modelo de exposição à fumaça de cigarro durante o período de lactação, a curto e longo prazo"**, sob a responsabilidade de **Patrícia Cristina Lisboa da Silva,** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), tendo sido aprovado pela Comissão de Ética Para o Cuidado e Uso de Animais Experimentais do Instituto de Biologia Roberto Alcântara Gomes da UERJ (CEUA), em **28/01/2014.** Este certificado expira em **28/01/2018.**

Rio de Janeiro, 28 de Janeiro de 2014.

Profa. Dra. Patricia C. Lisboa
CEUA/IBRAG/UERJ

Prof. Dr. Alex C. Manhães
CEUA/IBRAG/UERJ

Prof. Dr. Israel Felzenszwalb
CEUA/IBRAG/UERJ

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biologia@uerj.br

ANEXO B - Aprovação do Comitê de Ética Modelo experimental nicotina

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

ibrag Instituto de Biologia Roberto Alcantara Gomes

C E R T I F I C A D O

Certificamos que a proposta intitulada "**AVALIACOES CENTRAIS E PERIFÉRICAS EM RATOS PROGRAMADOS PELA EXPOSICAO MATERNA A NICOTINA NA LACTAÇÃO: ESTUDO MORFOFUNCIONAL DO HIPOTÁLAMO E DO TECIDO ADIPOSO MARROM**",
registrada com o nº 007/2017, sob a responsabilidade de **Patrícia Cristina Lisboa da Silva** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA) do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 31/01/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	31/01/2021
Espécie/linhagem/raça	Rato Wistar
Nº de animais	140
Peso/Idade	200-500 g / 90 – 180 dias
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 31 de Janeiro de 2017.

Prof. Dr. Alex C. Manhães
Coordenador
CEUA/IBRAG/UERJ

Profa. Dra. Patricia C. Lisboa
Vice-Coordenadora
CEUA/IBRAG/UERJ

http://www.biologiauerj.com.br/comite-de-etica
ceua.ibrag@yahoo.com.br

ANEXO C - Aprovação do Comitê de Ética Modelo experimental desmame precoce

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

C E R T I F I C A D O

Certificamos que a proposta intitulada "**INTERAÇÃO DO SISTEMA NERVOSO AUTÔNOMO E SISTEMA ENDOCANABINÓIDE EM RATOS PRECOCEMENTE DESMAMADOS**", registrada com o nº 035/2017, sob a responsabilidade de **Rosiane Aparecida Miranda** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)
do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 25/07/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	25/07/2021
Espécie/linhagem/raça	Rato Wistar
Nº de animais	220
Peso/Idade	200-600 g / 60 - 180 dias
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 25 de Julho de 2017.

Prof. Dr. Alex C. Manhães
Coordenador
CEUA/IBRAG/UERJ

Profa. Dra. Patricia C. Lisboa
Vice-Coordenadora
CEUA/IBRAG/UERJ

ANEXO D – Publicações artigos científicos coautora durante o período do doutorado

Journal Pre-proof

Programming of hepatic lipid metabolism in a rat model of postnatal nicotine exposure – Sex-related differences

Iala Milene Bertasso, Carla Bruna Pietrobon, Bruna Pereira Lopes, Thamara Cherem Peixoto, Patrícia Novaes Soares, Elaine Oliveira, Alex Christian Manhães, Maria Lucia Bonfleur, Sandra Lucinei Balbo, Suellen Silva Cabral, George Eduardo Gabriel Kluck, Georgia Correa Atella, Egberto Gaspar de Moura, Patrícia Cristina Lisboa

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journal homepage: www.elsevier.com/locate/lifescie

Cigarette smoke during lactation in rat female progeny: Late effects on endocannabinoid and dopaminergic systems

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ARTICLE INFO

Keywords:
Cigarette smoke
Endocannabinoids
Dopaminergic pathway
Metabolic programming
Breastfeeding

ABSTRACT

Aims: Maternal smoking is considered a risk factor for childhood obesity. In a rat model of tobacco exposure during breastfeeding, we previously reported hyperphagia, overweight, increased visceral fat and hyperleptinemia in adult female offspring. Obesity and eating disorders are associated with impairment in the endocannabinoid (EC) and dopaminergic (DA) systems. Considering that women are prone to eating disorders, we hypothesize that adult female Wistar rats that were exposed to cigarette smoke (CS) during the suckling period would develop EC and DA systems deregulation, possibly explaining the eating disorder in this model.

Material and methods: To mimic maternal smoking, from postnatal day 3 to 21, dams and offspring were exposed to a smoking machine, 4×/day/1 h (CS group). Control animals were exposed to ambient air. Offspring were evaluated at 26 weeks of age.

Key findings: Concerning the EC system, the CS group had increased expression of diacylglycerol lipase (DAGL) in the lateral hypothalamus (LH) and decreased in the liver. In the visceral adipose tissue, the EC receptor (CB1r) was decreased. Regarding the DA system, the CS group showed higher dopamine transporter (DAT) protein expression in the prefrontal cortex (PFC) and lower DA receptor (D2r) in the arcuate nucleus (ARC). We also assessed the hypothalamic leptin signaling, which was shown to be unchanged. CS offspring showed decreased plasma 17β-estradiol.

Significance: Neonatal CS exposure induces changes in some biomarkers of the EC and DA systems, which can partially explain the hyperphagia observed in female rats.

1. Introduction

Smoking is a risk factor for the onset of several diseases and is responsible for approximately 6 million annual deaths worldwide [1]. Tobacco is still the drug most commonly used by women during gestation and lactation, which are critical periods for fetus development, and it is known that cigarette components are transferred through placenta and breast milk, leading to negative consequences for the mother and possibly programming the baby's metabolism [2–4]. Metabolic programming can be caused by nutritional and/or environmental alterations during plasticity periods (pregnancy and breastfeeding) that cause epigenetic changes, increasing the risk of occurrence of diseases at adulthood, such as obesity [5,6]. In rodents, studies have already demonstrated that adult offspring from both sexes that were exposed to cigarette smoke exclusively during lactation were programmed for hyperphagia, overweight, increased visceral fat, hyperleptinemia and dyslipidemia [7,8]. In this experimental model, Pinheiro et al. (2015) observed an increase in the preference for a fat-rich palatable diet in males, which was attributed to changes in the dopaminergic system, such as lower dopamine production, consequently decreasing the effects of reward mechanisms [9].

Hyperphagia, obesity and metabolic programming have been associated with changes in the endocannabinoid (EC) system [10–12]. The EC system directly controls the central and peripheral mechanisms involved in energy homeostasis and food intake. EC action is mediated by the specific receptors, CB1r and CB2r, and their endogenous ligands, anandamide (AEA) and 2-aracdonoylglycerol (2-AG) [13–16]. The AEA is synthesized by *N*-acylphosphatidylethanolamide-phospholipase D

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[1] Equally contributed to this work.

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Alterations of the expression levels of CPT-1, SCD1, TRβ-1 and related microRNAs are involved in lipid metabolism impairment in adult rats caused by maternal coconut oil intake during breastfeeding

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ARTICLE INFO

Keywords:
Coconut oil
Metabolic programming
Obesity
Thyroid function
Lipid metabolism
MicroRNA

ABSTRACT

We studied the molecular and epigenetic mechanisms associated with dysfunctions caused by postnatal coconut oil exposure. Lactating dams were divided into soybean oil (SO) and coconut oil (CO) groups (0.5 g/kg/gavage). Half of the CO offspring received CO in their chow throughout their lifetime (CO + C). Adult CO offspring had higher liver TRβ-1 mRNA, which is consistent with lower miR-181a and lower DIO2 mRNA in brown adipose tissue (BAT) and higher CPT-1 mRNA in white adipose tissue (WAT). CO + C offspring exhibited higher BAT miR-382*, but DIO2 was unaltered. This group also had higher liver SCD1, miR-122 expression, and WAT SCD1 mRNA. CO and CO + C offspring had lower hepatic CPT-1 mRNA. The disturbances in CO offspring may be partially attributed to alterations in the enzymes involved in lipid metabolism, which may be mediated via mechanisms associated with miR-122. Continuous exposure to CO prohibits some of these changes.

1. Introduction

Data demonstrate a strong association between exposure to nutritional and environmental factors in critical periods of life, such as pregnancy and breastfeeding, with the emergence of metabolic diseases in adult life due to adaptive physiological changes in the organism. This phenomenon is called metabolic programming, but the mechanisms by which it occurs are not fully elucidated (Barker, 2003; Breier, Vickers, Ikenasio, Chan, & Wong, 2001; De Moura, Lisboa, & Passos, 2008). Programming is an epigenetic phenomenon that does not involve mutations or alterations of the DNA nucleotide sequence. Therefore, epigenetic alterations that occur early in life act as imprinting factors that may lead to structural and functional changes that may become permanent later in life (De Moura et al., 2008; Moura & Passos, 2005). Epigenetic alterations, such as DNA methylation, acetylation and methylation of histones and microRNAs (miRNAs)-mediated gene silencing, make an individual more adapted to his/her environment (Hirst & Marra, 2009). However, if the environmental conditions change during development, then the process may not be adaptive but increase the risk of metabolic disorders in adult life (De Moura et al., 2008).

Previous experimental studies showed that alterations in the dietary lipid profile early in life programmed adult offspring for obesity and the development of metabolic dysfunctions (Fernandes et al., 2012; Guarda et al., 2014, 2016; Magri et al., 2015; Quitete, Lisboa, Moura, & de Oliveira, 2018). An experimental model of metabolic programming in which lactating rats were supplemented with coconut oil during breastfeeding showed that the adult offspring exhibited overweight, high adiposity, hyperphagia, hyperleptinemia and thyroid dysfunctions. When these animals were subjected to coconut oil diet supplementation throughout life, most of these disorders were prevented, which suggests that this oil caused the disorders only when consumed in critical periods (Quitete, de Moura, Atella, Lisboa, & de Oliveira, 2019). However, the molecular and epigenetic mechanisms associated with the observed alterations were not elucidated. An increasing number of studies

Abbreviations: BAT, brown adipose tissue; CO, coconut oil group; CO+C, coconut oil + chow supplemented with coconut oil group; CPT-1, carnitine palmitoyltransferase-1; DIO, deiodinase; microRNA, miRNA; SCD1, stearoyl-CoA desaturase-1; SO, soybean oil group; T3, triiodothyronine; TSH, thyroid stimulating hormone; TRβ-1, thyroid hormone receptor beta-1; WAT, white adipose tissue

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Maternal coconut oil intake on lactation programs for endocannabinoid system dysfunction in adult offspring

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ABSTRACT

Maternal exposure to coconut oil metabolically programs adult offspring for overweight, hyperphagia and hyperleptinemia. We studied the neuroendocrine mechanisms by which coconut oil supplementation during breastfeeding as well as continued exposure of this oil throughout life affect the feeding behavior of the progeny. At birth, pups were divided into two groups: Soybean oil (SO) and Coconut oil (CO). Dams received these oils by gavage (0.5 g/kg body mass/day) during lactation. Half of the CO group continued to receive CO in chow throughout life (CO + C). Adult CO and CO + C groups had overweight; the CO group had hyperphagia, higher visceral adiposity, and hyperleptinemia, while the CO + C group had hypophagia only. The CO group showed higher DAGLα (endocannabinoid synthesis) but no alteration of FAAH (endocannabinoid degradation) or CB1R. Leptin signaling and GLP1R were unchanged in the CO group, which did not explain its phenotype. Hyperphagia in these animals can be due to higher DAGLα, increasing the production of 2-AG, an orexigenic mediator. The CO + C group had higher preference for fat and lower hypothalamic GLP1R content. Continuous exposure to coconut oil prevented an increase in DAGLα. The CO + C group, although hypophagic, showed greater voracity when exposed to a hyperlipidemic diet, maybe due to lower GLP1R, since GLP1 inhibits short-term food intake.

1. Introduction

Coconut oil is considered an edible oil with numerous therapeutic benefits. It is obtained from the pulp of the ripe and dried fruit, or coconut milk, resulting in the virgin form of the product (Kumar, 2011; Marina et al., 2009). This oil is mainly composed of medium-chain saturated fatty acids (MCSFAs), with lauric acid being the most predominant, approximately 48% (Lockyer, Stanner, 2016). The intake of medium-chain triglycerides (MCTs) is associated with lower food intake and lower body mass (Bach, Babayan, 1982; Ferreira et al., 2014; St-Onge, Jones, 2002). As coconut oil is rich in MCSFAs, it has been used indiscriminately as a substitute for traditional sources of fat in the Western diet, including for women during pregnancy and breastfeeding. However, it is known that transient changes in maternal nutrition during these critical periods may predispose to obesity and other diseases in the adult progeny (Bispo et al., 2015; Guarda et al., 2016; Moura, Passos, 2005; Oben et al., 2010; Qasem et al., 2016), a phenomenon known as metabolic programming.

Abbreviations: 2-AG, 2-arachidonoylglycerol; ARC, Arcuate nucleus; CB1R, Cannabinoid receptor type 1; CNS, Central nervous system; CO, Coconut Oil group; CO + C, Coconut oil + chow supplemented with coconut oil; D1-R, Dopaminergic receptor 1; D2-R, Dopaminergic receptor 2; DAGLα, Diacylglycerol lípase α; DAT, Dopamine transporter; DDc, DOPA decaborxylase; DS, Dorsal striatum; EC, Endocannabinoids; ECS, Endocannabinoid system; FAAH, Fatty acid amide hydrolase; GHSR-1a, ghrelin receptor; GI, gastrointestinal; GLP-1, Glucagon like peptide −1; GLP1R, Glucagon like peptide −1 receptor; HFD, High-fat diet; HSD, High-sugar diet; JAK2, Janus Kinase 2; LH, Lateral hypothalamus; MAGL, Monoacylglycerol lípase; MCH, Melanin-concentrating hormone; MCT, Medium chain triglycerides; NAc, Nucleus accumbens; Ob-R, Leptin receptor; PFC, Prefrontal córtex; PN, Post natal; SN, Substantia nigra; SO, Soybean Oil group; SOCS3, Supressor of cytokine signaling 3; STAT3, Signal transducer and activator of transcription 3; TH, Tyrosine hydroxylase; VFM, Visceral fat mass; VTA, Ventral tegmental área

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Article

Cigarette Smoke During Breastfeeding in Rats Changes Glucocorticoid and Vitamin D Status in Obese Adult Offspring

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Abstract: Maternal smoking increases obesogenesis in the progeny. Obesity is associated with several hormonal dysfunctions. In a rat model of postnatal tobacco smoke exposure, we previously reported increased central fat depot and disruption of some hormonal systems in the adult offspring. As both glucocorticoids and vitamin D alter lipogenesis and adipogenesis, here we evaluated the metabolism of these two hormones in visceral adipose tissue (VAT) and liver by Western blotting, and possible associations with lipogenesis biomarkers in adult rats that were exposed to tobacco smoke during their suckling period. At postnatal day (PN) 3, dams and offspring of both sexes were exposed (S group) or not (C group) to tobacco smoke, 4 × 1 h/day. At PN180, corticosteronemia was lower in S male and higher in S female offspring, without alterations in peripheral glucocorticoid metabolism and receptor. Adrenal ACTH receptor (MC2R) was higher in both sexes of S group. Despite unchanged serum vitamin D, liver 25-hydroxylase was higher in both sexes of S group. Male S offspring had higher 1α-hydroxylase, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) in VAT. Both sexes showed increased ACC protein content and reduced sirtuin mRNA in liver. Male S offspring had lower liver peroxisome proliferator-activated receptor-α. Tobacco exposure during lactation induced abdominal obesity in both sexes via distinct mechanisms. Males and females seem to develop HPA-axis dysfunction instead of changes in glucocorticoid metabolism and action. Lipogenesis in VAT and liver, as well as vitamin D status, are more influenced by postnatal smoke exposure in male than in female adult rat offspring.

Keywords: cigarette smoke; lactation; programming; adipose tissue; liver

1. Introduction

It is well established that smoking is a risk factor for the development of some chronic non-communicable diseases, such as respiratory diseases, some types of cancers, cardiovascular diseases, and glucose intolerance, in addition to being one of the major causes of early death

Supplementation of suckling rats with cow's milk induces hyperphagia and higher visceral adiposity in females at adulthood, but not in males

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ORIGINAL ARTICLE

Effects of cigarette smoke exposure during suckling on food intake, fat mass, hormones, and biochemical profile of young and adult female rats

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Abstract

Purpose Children from smoking mothers have a higher risk of developing obesity and associated comorbidities later in life. Different experimental models have been used to assess the mechanisms involved with this increased risk. Using a rat model of neonatal nicotine exposure via implantation of osmotic minipumps in lactating dams, we have previously shown marked sexual dimorphisms regarding metabolic and endocrine outcomes in the adult progeny. Considering that more than four thousand substances are found in tobacco smoke besides nicotine, we then studied a rat model of neonatal tobacco smoke exposure: adult male offspring had hyperphagia, obesity, hyperglycemia, hypertriglyceridemia, secondary hyperthyroidism and lower adrenal hormones. Since litters were culled to include only males and since sexual dimorphisms had already been identified in the nicotine exposure model, here we also evaluated the effects of tobacco smoke exposure during lactation on females.

Methods Wistar rat dams and their pups were separated into two groups of 8 litters each: SMOKE (4 cigarettes per day, from postnatal day 3 to 21) and CONTROL (filtered air). Offspring of both sexes were euthanized at PN21 and PN180.

Results Changes in male offspring corroborated previous data. At weaning, females showed lower body mass gain and serum triglycerides, but no alterations in visceral fat and hormones. At adulthood, females had higher body mass, hyperphagia, central obesity, hyperleptinemia, hypercholesterolemia, hypercorticosteronemia, but no change in serum TSH and T3, and adrenal catecholamine

Conclusions Sexual dimorphisms were observed in several parameters, thus indicating that metabolic and hormonal changes due to smoke exposure during development are sex-dependent.

Keywords Cigarette smoke · Lactation · Adipose tissue · Hormones, Female rats

Introduction

Worldwide, the prevalence of obesity is increasing markedly [1, 2]. Besides genetic factors, diet, and hormone alterations in critical windows of development permanently change some physiological parameters, contributing to increase the risk of emergence of diseases such as obesity, dyslipidemia, diabetes, and hypertension later in life. Currently this phenomenon is known as metabolic programming or developmental plasticity, and epigenetic changes have been implicated in its occurrence [3–5]. In addition, it is known that environmental alterations, such as exposure to cigarette smoke, act as programming factors, leading, for instance, to the development of obesity. Accordingly, epidemiological and clinical studies report that prenatal maternal smoking is a risk factor for child and adolescence obesity and hypertension [6–9].

Postpartum relapse has been consistently observed in lactating women who quit smoking cigarettes during

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ANEXO E - Publicações em revistas de divulgação sobre modelos da Tese

MÃES FUMANTES, FILHOS OBESOS

Estudo em animais mostra que tabagismo durante o período da amamentação aumenta a probabilidade de bebês apresentarem sobrepeso na vida adulta, mesmo quando as mães pararam de fumar na gestação.

A obesidade é uma epidemia que atinge o mundo todo. De acordo com a Organização Mundial da Saúde (OMS), os casos de obesidade mais do que dobraram nos últimos 30 anos. Em 2016, 41 milhões das crianças menores de 5 anos estavam acima do peso ou eram obesas no mundo, aumentando o risco de se tornarem adultos obesos e de desenvolverem desordens associadas ao sobrepeso, como dislipidemia – a popular 'gordura no sangue' – e diabetes. Pesquisa feita pelo Ministério da Saúde em 2018 indica que 12,9% das crianças brasileiras de 5 a 9 anos são obesas. Estudo recente realizado na Universidade do Estado do Rio de Janeiro (Uerj) revelou que mães que fumam durante o período de amamentação aumentam o risco de seus filhos se tornarem obesos na vida adulta.

Em uma pesquisa experimental com um modelo animal, investigamos os fatores que, durante a amamentação, podem aumentar o risco de desenvolver obesidade e suas doenças associadas, como diabetes, dislipidemias, hipertensão e doenças cardiovasculares. A surpresa veio na análise do fator tabagismo.

Constatamos, a partir desse experimento em ratos de ambos os sexos, que o tabagismo materno aumenta as chances de

Sabe-se que mudanças ambientais, nutricionais e hormonais em fases críticas do desenvolvimento, como a lactação, podem acarretar alterações epigenéticas (no funcionamento de genes) permanentes, deixando o indivíduo mais suscetível a manifestar sobrepeso e as complicações que dele decorrem. Esse fenômeno é definido como programação metabólica. Já foi demonstrado em animais que o desmame precoce é um fator de programação metabólica, uma vez que favorece, na idade adulta, o aumento de peso, a gordura corporal, alterações hormonais, resistência à leptina

seus filhotes se tornarem obesos na idade adulta

(hormônio produzido por células de gordura) e a redução da termogênese (processo metabólico durante o qual o corpo queima calorias para produzir calor).

Experimento em animais

Nosso grupo, sob orientação de Patrícia Cristina Lisboa e Egberto Gaspar de Moura, ambos Cientistas do Nosso Estado (CNE/Faperj), decidiu avaliar o que ocorre com mães que param de fumar na gestação, mas retomam o hábito durante a fase do aleitamento. Para isso, usamos dois modelos animais (ratos Wistar, incluindo mães e filhotes) de tabagismo materno na lactação. O primeiro, de exposição indireta e direta da prole à fumaça do cigarro: mães e filhos vão para a máquina de exposição ao cigarro; assim, o filhote é exposto por meio do leite materno (via indireta) e diretamente na máquina que simula um ambiente tabagista. O segundo, de exposição à nicotina, em que se implanta uma minibomba osmótica de nicotina nas mães lactantes e os filhotes são expostos exclusivamente através do leite materno.

Constatamos, a partir desse experimento em ratos de ambos os sexos, que o tabagismo materno aumenta as chances de seus filhotes se tornarem obesos na idade adulta, e que um dos mecanismos envolvidos nesse processo é a redução da capacidade termogênica (de aceleração do metabolismo) do tecido adiposo marrom (TAM). Os resultados foram publicados em 2018 no *Brazilian Journal of Medical and Biological Research*.

No modelo de exposição materna exclusiva à nicotina, também observamos uma redução da atividade do TAM. Em princípio, esse achado sugere que a nicotina seja o componente do cigarro que modula o processo de termogênese em nosso estudo.

O TAM é um tipo de tecido adiposo presente em mamíferos relacionado à regulação do calor (termogênese) e do gasto energético. A termogênese, por sua vez, é o mecanismo que permite dispersar energia sob forma de calor, processo que ocorre de acordo com as mudanças no meio externo ou com as necessidades metabólicas internas da célula.

Vários fatores podem interferir na termogênese, como exercícios físicos, dieta e temperatura ambiente. Até recentemente, acreditava-se que o TAM tinha relevância apenas para o recém-nascido, mas hoje já se sabe que esse tecido está presente em humanos adultos e que sua ativação e/ou restauração está relacionada a uma nova estratégia contra a obesidade.

A exposição à fumaça do cigarro no início da vida pode levar à disfunção do tecido adiposo marrom, comprometendo a capacidade termogênica dos filhotes quando adultos e, assim, favorecer o desenvolvimento da obesidade

Exposição à fumaça do cigarro

Outro achado do nosso estudo sobre o modelo de exposição à fumaça do cigarro está relacionado à inflamação do hipotálamo e à alteração de neuropeptídios importantes na regulação do balanço energético. Artigo sobre essa pesquisa foi publicado em setembro na revista *Neuroscience*.

O hipotálamo é uma região do cérebro que coordena grande parte das funções endócrinas, sendo importante na regulação da ingestão alimentar e do gasto energético. O processo de inflamação dessa região prejudica o seu funcionamento; portanto, a comunicação entre o hipotálamo e os tecidos periféricos (tecido adiposo branco, do pâncreas, da tireoide, entre outros) fica comprometida, favorecendo o desenvolvimento da obesidade.

Durante o doutorado, me interessei em estudar mais detalhadamente a função do tecido adiposo marrom, para compreender como este poderia impactar nas desordens metabólicas descritas pelo nosso grupo de pesquisa. Para isso, estudei, em animais, a atividade nervosa (simpática) associada ao TAM, analisando proteínas relacionadas à termogênese e identificando a forma dessas células de gordura em machos e fêmeas.

A grande novidade da pesquisa foi descrever que a exposição à fumaça do cigarro no início da vida pode levar à disfunção do tecido adiposo marrom, comprometendo a capacidade termogênica dos filhotes quando adultos e, assim, favorecer o desenvolvimento da obesidade. Essa descoberta tem grande relevância, uma vez que estudos com animais vêm apontando que o comprometimento da função do TAM está associado ao aumento de peso e gordura corporal, e que sua restauração e/ou ativação pode ser utilizada para terapias para combater a obesidade. Outro ponto importante são as diferenças entre sexos observadas, o que leva a uma melhor compreensão de como esses mecanismos ocorrem em homens e mulheres.

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Acreditamos que esses conhecimentos no campo da pesquisa básica em animais permitam um melhor entendimento das doenças prevalentes em seres humanos. E esperamos que eles possam guiar as autoridades públicas a criarem novas políticas para alertar as mulheres fumantes sobre os riscos para seus bebês durante a amamentação, mesmo que fumem longe de seus filhos; ajudar a entender melhor quais mecanismos estão envolvidos na obesidade e prevenir ou tratar adequadamente os distúrbios tardios.

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Matéria publicada em 19.11.2019

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Mãe fumante, filho obeso

Foto: LMoonlight/Pixabay

Estudo desenvolvido na Uerj mostra que o tabagismo materno no período de amamentação aumenta as chances do bebê se tornar um adulto obeso

Débora Motta

Os malefícios do cigarro e da obesidade já são amplamente conhecidos. Mas o que poucos sabem é que o tabagismo das mães durante o período da amamentação, após o término da gravidez, aumenta a probabilidade da criança apresentar obesidade na vida adulta. É o que demonstrou um estudo desenvolvido na Universidade do Estado do Rio Janeiro (Uerj), pela nutricionista Thamara Cherem Peixoto, que cursa o doutorado no Programa de Pós-Graduação em Biociências com apoio da FAPERJ, por meio do programa Bolsa Nota 10. Ela é orientada na pesquisa pela bióloga Patrícia Cristina Lisboa, contemplada, por sua vez, pelo programa Cientista do Nosso Estado, também da Fundação, e conta com co-orientação do professor Egberto Gaspar de Moura, que foi sub-reitor de Pós-Graduação e Pesquisa da Uerj entre 2016 e 2019.

O estudo foi realizado em um modelo animal, de ratos Wistar – incluindo mães e filhotes –, no Laboratório de Fisiologia Endócrina da Uerj. De acordo com os resultados obtidos com a pesquisa, um dos fatores que explicam essa obesidade é a redução da capacidade termogênica do tecido adiposo marrom dos filhotes. "Vimos que uma disfunção no tecido adiposo marrom dos filhotes, relacionada à redução da atividade simpática do nervo que vai para esse tecido, compromete a atividade termogênica, favorecendo o acúmulo de gordura corporal", explicou Thamara.

Ela lembrou que o tecido adiposo, além de ser o principal reservatório energético do organismo, é um centro regulador do metabolismo. "Uma das funções do tecido adiposo marrom é a termogênese, que é a regulação da temperatura corporal, relacionada ao gasto diário de energia do indivíduo. Quando reduzida, o metabolismo basal fica mais lento e a tendência é engordar", detalhou. "Outra alteração que explica a obesidade é a inflamação hipotalâmica e alteração de neuropeptídios importantes na regulação da fome e do gasto de energia", completou.

Foram realizados, simultaneamente, experimentos para observar os efeitos da exposição direta e indireta à fumaça do cigarro (mães e filhos). A exposição direta simulou a criança lactente no ambiente tabagista, exposta à fumaça. Já a exposição indireta, a criança que mama em uma mãe fumante, absorvendo a nicotina pelo leite materno. "Cada experimento durou cerca de oito meses dentro do biotério e envolveu etapas de acasalamento, gestação (três semanas), lactação (três semanas), programação, que foi o período de submissão dos filhotes à fumaça (26 semanas), além do período dedicado as análises (seis meses)", contou. No trabalho, a exposição à fumaça correspondeu àquela gerada por fumantes humanos moderados, que consomem em torno de 20 cigarros por dia, sendo que cada cigarro contém 0.73 mg de nicotina.

Foto: Reprodução

Um dos fatores que explicariam essa relação é a redução da capacidade termogênica do tecido adiposo marrom dos filhos

Foto: Divulgação

Os cigarros usados na pesquisa têm uma concentração de nicotina de 0.73 mg cada. Nos testes, a exposição à fumaça correspondeu àquela gerada por fumantes moderados

Diante da atual epidemia de obesidade, que atinge tanto a população de países desenvolvidos como de países em desenvolvimento, e dos prejuízos globais causados pelo tabagismo, a pesquisa pode ser um ponto de apoio para a formulação de políticas públicas para conscientizar a população. "Muitas mães param de fumar durante a gestação, mas voltam durante a lactação, sem saber dos riscos que podem causar. Esse é o ponto que queremos mostrar. A exposição ao metabólito da nicotina via leite materno acarreta problemas futuros para os filhos", alertou.

Tema da tese de doutorado de Thamara a ser defendida em agosto de 2020, o estudo já resultou na publicação de dois artigos em periódicos científicos internacionais. O primeiro artigo, *Neonatal tobacco smoke reduces thermogenesis capacity in brown adipose tissue in adult rats*, saiu em janeiro de 2018 no *Brazilian Journal of Medical and Biological Research*. E o segundo, intitulado *Hypothalamic neuropeptides expression and hypothalamic inflammation in adult rats that were exposed to tobacco smoke during breastfeeding: sex-related differences*, foi publicado em setembro de 2019 na revista *Neuroscience*, da International Brain Research Organization (IBRO).

Pesquisadora: Thamara Cherem Peixoto
Instituição: Universidade do Estado do Rio de Janeiro (Uerj)
Fomento: *Programa Bolsa Nota 10*

Foto: Divulgação/Uerj

Thamara, no Laboratório de Fisiologia Endócrina da Uerj, junto ao bioamplificador que faz a leitura do tecido adiposo marrom