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GLAUBER RUDÁ FEITOZA BRAZ

AVALIAÇÃO CENTRAL E PERIFÉRICA DA BIOENERGÉTICA MITOCONDRIAL, ESTADO REDOX E EXPRESSÃO DE MODULADORES DO BALANÇO ENERGÉTICO CORPORAL EM RATOS SUPERNUTRIDOS TRATADOS COM FLUOXETINA

Recife
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Tese apresentada ao Programa de Pós-graduação em Neuropsiquiatria e Ciências do Comportamento do Centro de Ciências da Saúde, Universidade Federal de Pernambuco como parte dos requisitos parciais para obtenção do título de Doutor em Neurociência.

Área de concentração: Neurociências

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RESUMO

A obesidade infantil atingiu proporções epidêmicas em todo o mundo e como consequência, várias disfunções metabólicas estão associadas a tal condição. A serotonina desempenha funções diversificadas no organismo, entre elas a de regulação do balanço energético corporal especialmente no sistema nervoso central (SNC) onde atua na integração de sinais centrais e periféricos relacionados à fome e saciedade. Metabolicamente importante, a função mitocondrial relacionada à produção de energia representa um ponto-chave no balanço energético, principalmente em condições de desequilíbrio metabólico característico do sobrepeso/obesidade. Entretanto, uma compreensão mais integrativa da relação entre o sistema serotoninérgico e a função mitocondrial associados ao balanço energético ainda se faz necessário. Assim, o presente trabalho objetivou investigar os efeitos da administração crônica com fluoxetina sobre a bioenergética mitocondrial, balanço oxidativo e moduladores do balanço energético corporal no hipotálamo e tecidos adiposos branco e marrom de ratos jovens supernutridos durante a lactação, por redução de ninhada. Para tal, ratos *Wistar* machos foram divididos ao 3º dia de vida em dois grupos: Normonutrido (n=9, por ninhada) e Supernutrido (n=3, por ninhada), n total = 27 por grupo. Aos 39 dias de vida, os grupos foram subdivididos conforme a administração de solução salina (NaCl, 0,9%) ou Fluoxetina (10mg/kg). Aos 60 dias de vida, foi avaliado o consumo de oxigênio mitocondrial, a produção de espécies reativas mitocondriais, biomarcadores de peroxidação lipídica e oxidação proteica, estado REDOX, Oxy-score e expressão de moduladores do balanço energético corporal no hipotálamo e tecidos adiposos. Observamos que ratos supernutridos apresentaram prejuízo na capacidade respiratória mitocondrial, com maior produção de espécies reativas e reduzido balanço REDOX, tanto no hipotálamo quanto no tecido adiposo marrom (TAM). A fluoxetina foi capaz de reverter a disfunção mitocondrial e estresse oxidativo, observada em animais supernutridos, em ambos os tecidos, mas não em animais normonutridos. Em relação ao perfil molecular, ratos supernutridos apresentaram aumento na expressão de leptina no tecido adiposo branco (TAB) e diminuição no tecido adiposo marrom (TAM), porém nenhuma alteração significativa nas análises hipotalâmicas. Entretanto, a administração com fluoxetina em ratos

supernutridos foi capaz de induzir aumento da expressão gênica da proteína desacopladora mitocondrial (UCP) 2 no hipotálamo, assim como de moduladores do processo de diferenciação do tecido adiposo branco em marrom (*browning*) e de biogênese mitocondrial no hipotálamo. Portanto, de maneira geral nossos resultados sugerem que a administração crônica com fluoxetina em ratos supernutridos está associada a uma melhor capacidade funcional das mitocôndrias e um melhor balanço oxidativo, bem como uma adaptação molecular que favorece o dispêndio energético e a biogênese mitocondrial, regulando de maneira positiva o balanço energético corporal, do ponto de vista de eficiência mitocondrial energética, em ratos supernutridos.

Palavras-chave: Fluoxetina. Hipotálamo. Mitocôndrias. Obesidade. Tecido adiposo marrom.

ABSTRACT

The childhood overweight/obesity has become increasingly higher in the past years and hence several metabolic dysfunctions are associated with such condition. Serotonin plays a dynamic role in the organism and in the central nervous system coordinates the energy balance regulation inducing hunger or satiety mechanisms. Mitochondrial function related to energy production remains a key factor in energy balance regulation mainly in conditions of metabolic impairments such as overweight/obesity. However, a more integrative comprehension of the relationship between the serotoninergic system and the mitochondrial function associated with energy balance still is not totally understood. Thus, the present study aimed to evaluate the effects of chronic fluoxetine treatment on mitochondrial bioenergetics, oxidative balance and modulatory genes of the body energy balance in the hypothalamus and white and brown adipose tissues of postnatal overfed rats (using litter size reduction). To address this concern, male *Wistar* rats were assigned into two groups at the postnatal day (PND) 3: Normofed (n=9 per litter) and Overfed (n=3 per litter), n=27 per group. At PND 39, the groups were subdivided according to administration of vehicle solution (NaCl, 0.9%) or Fluoxetine (10mg/kg). At PND 60, we evaluated mitochondrial oxygen consumption, mitochondrial reactive species production, lipid peroxidation, protein oxidation, REDOX state, Oxy-score and molecular regulators of energy balance in the hypothalamus and adipose tissues. Overfed rats showed impaired mitochondrial respiratory capacity, higher production of reactive species and reduced REDOX status in both hypothalamus and interscapular brown adipose tissue (iBAT). Fluoxetine was able to reverse mitochondrial dysfunction and oxidative stress in the hypothalamus and iBAT of overfed rats. Regarding to the molecular profile, overfed rats showed increased expression of leptin in the white adipose tissue (WAT) and reduced expression in the interscapular brown adipose tissue (iBAT) adipose tissues but no significant alteration in hypothalamic analyzes. In addition, fluoxetine administration in overfed rats triggered higher gene expression of mitochondrial biogenesis regulators in the hypothalamus and induced browning adipogenesis programming in WAT. Overall, our results suggest that chronic modulation of serotonin system in overfed rats improves mitochondrial function and oxidative

balance, as well as the molecular pathways that favors energy expenditure and mitochondrial biogenesis, improving body energy balance towards bioenergetic efficiency in overfed rats.

Keywords: Brown adipose tissue. Fluoxetine. Hypothalamus. Mitochondria. Obesity. Serotonin.

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5-HT	5-Hidroxitriptamina, Serotonina
ADP	Adenosina Difosfato
AgRP	Peptídeo Relacionado ao Gene Agouti
AMPK	Proteína Kinase Dependente de AMP
ATP	Adenosina Trifosfato
ATP5A	Subunidade Alfa da ATP Sintetase
BSA	Albumina do Soro Bovino
Ca^{2+}	Íon Cálcio
CART	Transcrito Relacionado à Cocaína e Anfetamina
CCCP	Carbonilcianeto-3-clorofenil-hidrazona
DNPH	2,4-Dinitrofenilhidrazina
EDTA	Ácido Etilenodiamino tetra-ácido
EGTA	Ácido Etileno glicol-bis(2-aminoetIl)-N,N,N′,N′-tetra-acético
EROS	Espécies Reativas de Oxigênio
FLX	Fluoxetina
GDP	Guanosina 5′-difosfato de sódio
GSH	Glutationa Reduzida
GSSG	Glutationa Oxidada
GST	Glutationa-S-transferase
HEPES	Hidroxietil piperazina ácido etanesulfonico
IMC	Índice de Massa Corporal
KCL	Cloreto de Potássio
KH_2PO_4	Fosfato de Potássio Monobásico
MAO	Monoamina Oxidase
MCR	Receptor de melanocortina
MDA	Malondialdeído
NADH	Dinucleotídeo de Nicotinamida e Adenina
NaOH	Hidróxido de sódio
NDUFB8	Subunidade B8 do complexo mitocondrial I
NPY	Neuropeptídeo Y
NRF1	Fator respiratório nuclear 1

NRF2	Fator respiratório nuclear 2
OMS	Organização Mundial da Saúde
PCR	Reação em Cadeia da Polimerase
PGC1α	Coativador 1 de PPAR gama
PMSF	Fluoreto de Fenilmetanossulfonil
POMC	Proopiomelanocortina
PRDM16	Proteína domínio PR 16
PTPM	Poro de Transição de Permeabilidade Mitocondrial
SDHB	Subunidade B do complexo mitocondrial II
SERT	Transportador de Serotonina
SIRT1	Sirtuína 1
SNC	Sistema Nervoso Central
SOD	Superóxido Dismutase
SSRI	Inibidores Seletivos de Recaptação de Serotonina
TAB	Tecido Adiposo Branco
TAM	Tecido Adiposo Marrom
TBARS	Substância Reativa ao Ácido Tiobarbitúrico
TFAM	Fator de transcrição mitocondrial A
TPH1	Triptofano Hidroxilase 1
TPH2	Triptofano Hidroxilase 2
UCP1	Proteína desacopladora mitocondrial 1
UCP2	Proteína desacopladora mitocondrial 1
WOF	Federação Mundial de Obesidade

SUMÁRIO

1 INTRODUÇÃO

Segundo a Organização Mundial de Saúde (OMS), um ambiente nutricional neonatal desequilibrado tem contribuído para que a obesidade infantil alcance índices alarmantes no cenário mundial e que estão fortemente associados ao aparecimento de doenças crônico-degenerativas na vida adulta. Dados recentes publicados pela Federação de Combate à Obesidade no Mundo revelou que, em 2030, mais de 250 milhões de crianças estarão obesas (WOF, 2019). A obesidade ou sobrepeso do ponto de vista metabólico é um desequilíbrio entre a quantidade de energia (calorias) consumida na alimentação e a quantidade de energia gasta (dissipada) ao longo do dia. Esse controle energético é processado no encéfalo a partir de sinais periféricos da adiposidade e saciedade, que modificam vias anabólicas e catabólicas, e consequentemente, alteram o balanço energético de acordo com a necessidade (ABIZAID e HORVATH, 2012; KEEN-RHINEHART, et al., 2013). Nesse sentido, o sistema serotoninérgico encefálico, em especial, participa substancialmente dessa relação (HEISLER, et al., 2006; VICKERS, et al., 2008) do ponto de vista neurobiológico e comportamental, modulando vias importantes na regulação do consumo alimentar mediado principalmente pelo hipotálamo. Atuando no sistema nervoso central (SNC), a serotonina possui atuações diversificadas referentes à função regulatória do comportamento, da imunidade, da resposta pressórica, entre outros (WATTS, et al., 2012; SANCHEZ, et al., 2015; SHAJIB e KHAN, 2015). Entretanto, pouco se sabe acerca da influência serotoninérgica sobre o metabolismo energético relacionado à modulação da bioenergética mitocondrial e fatores associados a esse balanço energético de maneira central e periférica.

As mitocôndrias, classicamente conhecidas por seu papel incontestável na formação de energia celular, têm sido alvo de incontáveis estudos pelo seu papel determinante na regulação da homeostase energética, balanço REDOX, e mecanismos de interação intracelular e intercelular (HALLIWELL e GUTTERIDGE, 1986; FIGUEIRA, et al., 2013; VERCESI, et al., 2018). Entretanto, essa organela pode contribuir em funções usualmente distintas da bem estabelecida produção de energia química (na forma de trifosfato de adenosina-ATP). A exemplo, encontrada em abundância no tecido adiposo

marrom, as mitocôndrias atuam com uma especificidade direcionada para dissipar a energia derivada do metabolismo de substratos em forma de calor (TRAYHURN, 2017). Porém, independente do tecido estudado, a regulação metabólica exercida pelas mitocôndrias está associada à produção de espécies reativas de oxigênio (EROS), que quando em excesso, favorecem um estado pró-oxidante celular (TAHARA, et al., 2009). Dessa maneira, estudos têm demonstrado que disfunções mitocondriais contribuem em processos fisiopatológicos, sugerindo que o desenvolvimento de diversas doenças e/ou o agravamento de desequilíbrios bioquímicos e fisiológicos pré-existentes podem ocorrer de maneira dependente da capacidade adaptativa mitocondrial aos estímulos estressores submetidos à mesma (PICARD, et al., 2015; LAHERA, et al., 2017; ANGELOVA e ABRAMOV, 2018).

O potencial modulatório da serotonina em mecanismos associados à melhora do balanço energético do ponto de vista mitocondrial representa uma lacuna na literatura. Clinicamente importante no tratamento de distúrbios neurológicos como depressão e ansiedade, inibidores seletivos de recaptação de serotonina (ISRS) atuam bloqueando a proteína transportadora de serotonina (SERT) no neurônio pré-sináptico, aumentando as concentrações da mesma na fenda sináptica e sua consequente interação com os receptores serotoninérgicos pós-snápticos (MASAND e GUPTA, 1999; KRISHNAN e NESTLER, 2008). Estudos sobre a associação dos ISRS com o balanço energético têm evidenciado um comportamento hipofágico e redução em índices murinométricos em roedores, a depender do tempo e dose de exposição ao fármaco (SIMANSKY, 1996; DA SILVA, et al., 2019). Dessa maneira, esse estudo pode contribuir para uma maior compreensão integrativa dessa temática, associada ao sobrepeso/obesidade.

A esse respeito, o tratamento crônico com fluoxetina em animais supernutridos durante a lactação conduziu a presente tese a testar as seguintes hipóteses: (1) a supernutrição pós-natal prejudica o balanço energético corporal por disfunção mitocondrial e aumento de estresse oxidativo no hipotálamo e tecido adiposo marrom de ratos *Wistar* machos aos 60 dias de vida, e (2) o tratamento crônico com fluoxetina em ratos jovens melhora a atividade mitocondrial e balanço oxidativo, além de favorecer a expressão de moduladores

do balanço energético corporal no hipotálamo e tecidos adiposos branco e marrom de ratos *Wistar* machos, aos 60 dias de vida.

Como principais resultados, esta tese originou três artigos científicos. O artigo de revisão sistemática intitulado *"Systematic review of serotonin reuptake inhibitors (SSRI) effects on the rat brain mitochondria"* foi submetido à revista Brain Research - Qualis B1 (Anexo B). Esse trabalho teve como principais objetivos a síntese de estudos, através de uma estratégia de pesquisa sistemática para seleção e coleta de dados, relacionados aos efeitos de inibidores de recaptação de serotonina sobre a função mitocondrial de regiões encefálicas em modelos experimentais (ratos) de doenças do SNC, bem como a identificação dos potenciais mecanismos subjacentes associados à essa modulação farmacológica da função mitocondrial.

O artigo original intitulado "*Serotonin modulation in overfed rats improves hypothalamic mitochondrial respiration, reduces oxidative stress and induces mitochondrial biogenesis*" foi submetido à revista European Journal of Pharmacology - Qualis B1 (Anexo C) e objetivou avaliar os efeitos do tratamento crônico com fluoxetina em animais supernutridos sobre a função mitocondrial, balanço oxidativo e indicadores de biogênese mitocondrial no hipotálamo, importante centro regulador do balanço energético corporal.

O segundo artigo original da tese intitulado "*Chronic serotonin reuptake inhibition uncouples brown fat mitochondria and induces beiging/browning process of white fat in overfed rats*" foi aceito para publicação na revista Life Sciences - Qualis B1 (Anexo D) e corroborou nossos achados prévios de indução de um fenótipo propenso à eficiência metabólica em ratos supernutridos tratados com fluoxetina. Como principais novidades, o terceiro artigo desta tese demonstrou que a inibição crônica de serotonina em animais supernutridos não somente induz um maior desacoplamento mitocondrial independente de UCP no tecido adiposo marrom, como também favorece uma maior expressão de genes envolvidos na diferenciação molecular do tecido adiposo branco em bege/marrom.

2 REFERENCIAL TEÓRICO

2.1 Obesidade infantil e predisposição à comorbidades na vida adulta

A obesidade (do latim, *obesus*, gordura) é decorrente de uma maior proliferação dos precursores dos adipócitos (hiperplasia) e/ou do aumento no volume das células adiposas (hipertrofia) causado por uma excessiva ingestão calórica (MONTARANI, 2016). O índice de massa corporal (IMC) utilizado para avaliar o peso corporal (kg) em relação à estatura (m) e idade, classifica os indivíduos com sobrepeso quando apresentam valores acima de 25kg/m² para adultos e acima do percentil 85 para crianças e adolescentes entre 2 e 18 anos, e obesos quando o índice é maior que 30kg/m² para adultos e maior ou igual ao percentil 95 para indivíduos entre 2 e 18 anos; representando um caráter inicial da avaliação da massa corporal e composição corporal, de maneira genérica (FITCH, et al., 2013; JENSEN, et al., 2014).

Mundialmente, a obesidade representa uma ameaça à saúde de crianças e adolescentes e à sua qualidade de vida ao longo dos anos. Mais de 40 milhões de crianças e adolescentes encontram-se nessa condição, que é considerada uma doença desde 2013 por várias sociedades e organizações mundiais (WHO, 2018). Até 2016, 1 em cada 5 crianças e adolescentes no mundo encontrava-se inserida nos índices globais de obesidade infantil. Esses índices têm triplicado desde 1975, e segundo a Federação de Combate à Obesidade no Mundo tende a aumentar em mais de 250 milhões na próxima década (WOF, 2019).

O rápido e crescente aumento nos índices de obesidade globais concentrou-se inicialmente em países subdesenvolvidos e começou a ter uma notória preocupação das organizações de saúde com o reconhecimento da associação entre a alteração dietética da população e o aumento de doenças como diabetes e hipertensão, especialmente a partir da década de 90 (POPKIN, et al., 2012). A exposição ao ambiente obesogênico no início da vida tem sido relevante na prevalência de excesso de peso no Brasil, onde a mesma já é considerada pelo menos três vezes maior do que a prevalência de subnutrição (CONDE e MONTEIRO, 2014). Este fato é decorrente de uma transição nutricional mundial caracterizada pela inversão dos padrões de distribuição de problemas nutricionais anteriormente atribuídos à restrição alimentar e ao baixo

peso corporal, especialmente no nosso país onde as disparidades econômicas regionais são mais acentuadas e comprometiam em maior grau as regiões Norte e Nordeste (SOUSA, et al., 2016).

Diversos fatores contribuíram para essa alteração do padrão nutricional, como o aumento da acessibilidade a alimentos ultraprocessados, mais palatáveis e com alta densidade energética; associados ao aumento da renda familiar, o crescimento populacional, a diminuição na natalidade e a maior concentração populacional em áreas urbanas (MONTEIRO, et al., 1995; CONDE e MONTEIRO, 2014). Dessa maneira observa-se uma ascensão do sobrepeso/obesidade infantil e a perspectiva de aumento rápido desses índices que não mais são atribuídos primordialmente à realidade socioeconômica.

A obesidade possui um caráter multifatorial envolvido e as influências ambientais como a nutrição não são exclusivas para o desenvolvimento desta doença, que possui relação ainda com fatores biológicos, psicossociais e comportamentais (APOVIAN, 2016). Mesmo assim, o excesso de calorias e o menor dispêndio energético representam o principal binômio associado ao aumento da prevalência de doenças crônicas na vida adulta que reduzem a expectativa de vida e elevam o risco de morbidade e mortalidade. Entre as principais comorbidades associadas à obesidade encontram-se doenças cardiovasculares, musculoesqueléticas, neurodegenerativas, metabólicas e neoplásicas em geral (WHO, 2014; APOVIAN e RIFFENBURG, 2017). Sendo assim, tida como um desafio no atual cenário de má qualidade nutricional, a resolução conhecida como “década da nutrição” (2016-2025) pelas Nações Unidas (ONU, 2016) prioriza um planejamento de segurança alimentar e nutricional mais saudável e sustentável para o combate ao sobrepeso e obesidade. Essas ações representam, portanto, não somente uma redução nos custos diretos em cuidados com a saúde pública, mas principalmente uma perspectiva de melhoria na qualidade de vida e redução dos índices de mortalidade por doenças crônicas, tornando necessário o entendimento mais profundo dessa temática.

Entre os modelos de indução de sobrepeso em modelos experimentais encontra-se o modelo de supernutrição pós-natal por redução de ninhada (*small*

litter – SL), amplamente estudado pelo grupo de Plagemman (PLAGEMANN, et al., 1992; PLAGEMANN e HARDER, 2005; PLAGEMANN, 2011; PLAGEMANN, et al., 2012) e que busca mimetizar o consumo de uma alta densidade energética através da alimentação em excesso que mantém o fenótipo obeso na vida adulta, a exemplo do que constata-se entre crianças e adolescentes em todo o mundo. Diversos grupos demonstraram efeitos deletérios decorrentes desse tipo de insulto nutricional na lactação em modelos experimentais, como deficiência de crescimento e hipofuncionalidade de adipócitos marrons (MEDVEDEV e ELSUKOVA, 1999; DE ALMEIDA, et al., 2013), desregulação na expressão de neuropeptídeos hipotalâmicos que controlam o consumo alimentar (LOPEZ, et al., 2005; DA SILVA, et al., 2019), disfunção cardiovascular (HABBOUT, et al., 2013; JUNIOR, et al., 2019), deficiência na sinalização de leptina (RODRIGUES, et al., 2009), resistência à insulina (BEI, et al., 2015) entre outras disfunções metabólicas.

De maneira a investigar essa associação entre o ambiente obesogênico e o comprometimento metabólico, estudo recente do nosso laboratório demonstrou que uma supernutrição pós-natal em ratos machos induz aumento de adiposidade visceral, níveis séricos de glicose e triglicerídeos e comportamento hiperfágico aos 60 dias de vida (DA SILVA, et al., 2019). Além disso, esse modelo de supernutrição neonatal em ratos induziu dano glomerular condizente com uma possível redução da integridade e função renal aos 30 dias de vida (PEDROZA, et al., 2019) e maior área de infarto do miocárdio após evento de isquemia e reperfusão aos 60 dias de vida (DE MOURA FREITAS, et al., 2018). Em idades mais avançadas, a supernutrição pós-natal foi capaz de induzir fibrose cardíaca, hipertrofia ventricular e aumento da pressão arterial em ratos machos aos 120 dias (JUNIOR, et al., 2019). Em conjunto, essas evidências trazem à tona os efeitos deletérios do consumo alimentar em excesso, no início da vida, sobre a função tecidual, e a predisposição à disfunção metabólica e comorbidades associadas, que persistem em idades mais avançadas.

2.2 Balanço energético corporal: cooperação de vias centrais e periféricas e papel da serotonina

Se tratando do desenvolvimento da obesidade, fortes evidências demonstram que o excesso de peso característico dessa condição se deve em grande parte à desregulação dos mecanismos de controle do balanço energético, realizados majoritariamente no encéfalo (BILLINGTON, et al., 1994; COWLEY, et al., 2001; MCNAY, et al., 2012). Esse controle energético é processado a partir de sinais da adiposidade e saciedade provenientes da periferia, bem como de parâmetros mecânicos como a distensão gástrica, que em conjunto modificam tanto vias anabólicas como catabólicas, e consequentemente, alteram o comportamento de ingestão alimentar de acordo com o requerimento energético (WANG, et al., 2008; ABIZAID e HORVATH, 2012; KEEN-RHINEHART, et al., 2013; KIM, et al., 2018). O status metabólico portanto, decorrente da disponibilidade energética em termos de utilização e estoque, convém de uma associação entre sinalização nutricional, endócrina e neural (ZELTSER, 2018).

De maneira integrativa, a interpretação (sinalização) dos estímulos nutricionais envolvida no comportamento alimentar é comandada por diversas vias neurais que envolvem principalmente o hipotálamo e a comunicação com o tronco encefálico e sistema límbico (GRILL, 2010). Essas estruturas orquestram a regulação da homeostase energética mediada, por exemplo, pelo nível hormonal de leptina e grelina, envolvidos respectivamente na sinalização da saciedade a partir do tecido adiposo e da fome através do trato gastrointestinal, além de outros hormônios periféricos que influenciam a função neural como insulina e colecistocinina, também liberados no plasma em resposta à disponibilidade energética (AMIN e MERCER, 2016). O neurocircuito envolvido nessa regulação da homeostase energética possui projeções bem estabelecidas de vias aferentes e eferentes que regulam o balanço energético em sincronia, e o sistema serotoninérgico cerebral, em especial, participa substancialmente dessa relação (HEISLER, et al., 2006; VICKERS, et al., 2008).

Intimamente relacionado com a homeostase energética, o neurotransmissor serotonina (5-hidroxitriptamina ou 5-HT) é sintetizado a partir

do aminoácido essencial triptofano, com ação principal da enzima triptofano hidroxilase (TPH), e está envolvido na regulação de diversos processos fisiológicos e comportamentais (TECOTT, 2007). Notavelmente, a 5-HT possui importância crítica no neurodesenvolvimento, sendo um dos primeiros neurotransmissores encontrados em fases embrionárias de roedores (cerca de 12-13 dias após o início da embriogênese) (LAUDER e BLOOM, 1974; LAMBE, et al., 2000) e de humanos (5ª semana embrionária) (GINGRICH, et al., 2017), por servir como um importante fator de crescimento celular durante essa fase, associada a outros fatores neurotróficos e mensageiros químicos (SODHI e SANDERS-BUSH, 2004). Terminais serotoninérgicos desenvolvidos podem influenciar, portanto, na neurogênese, refinamento dendrítico, migração celular e plasticidade sináptica. Sua concentração possui variações durante todo o curso da vida, apresentando-se aumentada até o segundo ano de vida com posterior redução e estabilização de seus níveis aos 5 anos de idade, similar às concentrações encontradas na idade adulta, onde seu papel como neurotransmissor é então mais consolidado (SODHI e SANDERS-BUSH, 2004).

A contribuição da 5-HT no balanço energético é associada ao local de produção, que por vias periféricas é sintetizada em cerca de 95% pela TPH 1 nas células enterocromafins e neurônios do sistema nervoso entérico e é relacionada ao fenótipo obeso (CRANE, et al., 2015). Já no SNC, a TPH 2 sintetiza serotonina nos núcleos da rafe do tronco encefálico e coordena o efeito hipofágico e, portanto, antagônico à função periférica; especificidade adquirida pela incapacidade da 5-HT em atravessar a barreira hematoencefálica (NAMKUNG, et al., 2015).

Embora a serotonina demonstre uma atuação em diversas áreas centrais, o hipotálamo, que corresponde a conjuntos de corpos de neurônios dispersos em uma rede de substância branca (conjunto de axônios), permanece como o centro integrador do balanço energético mais essencial dos sinais de saciedade ou de fome provenientes de tecidos periféricos, tais como o tecido adiposo (MORTON, et al., 2006). Seu desenvolvimento possui fases bem estabelecidas, particularmente em ratos, para neurogênese (período embrionário), diferenciação celular (fim do período embrionário até 15 dias após o nascimento) e formação das sinapses (máxima resposta observada aos 35 dias pós-natal)

(BOURET, 2017). Todas elas são fases de alta vulnerabilidade a estímulos ambientais e potencialmente susceptíveis a efeitos irreversíveis sobre a estrutura e função do tecido (LUCAS, et al., 1984).

Uma das principais regiões hipotalâmicas envolvidas no controle alimentar é o núcleo arqueado, localizado no diencéfalo, imediatamente adjacente à eminência mediana. Possui uma população de neurônios onde se co-expressa os peptídeos proopiomelanocortina (POMC) e o transcrito relacionado à cocaína e anfetamina (CART); importantes reguladores do balanço energético pelas vias serotoninérgicas que favorecem o menor consumo alimentar e a saciedade (CONE, et al., 2001). Além destes, outras populações de neurônios, tais como as que co-expressam o neuropeptídeo Y (NPY) e o peptídeo relacionado ao gene agouti (AgRP), modulam o fluxo simpático a partir do hipotálamo para órgãos-alvo e estão associados a hiperfagia (CONE, et al., 2001; RODRIGUEZ, et al., 2010). Essas populações de neurônios expressam receptores serotoninérgicos do tipo 5HT1B e 5HT2C que modulam sua atividade em função da disponibilidade de serotonina e do estado energético. A mediação da atividade desses neuropeptídeos antagônicos e a resposta final sobre o consumo alimentar é realizada pelo sistema de melanocortina hipotalâmico, que compreende o produto da clivagem do POMC, o hormônio estimulante de alfa melanócito (α-MSH), e os receptores de melanocortina (MCR) (HEISLER, et al., 2006). Assim sendo, a ativação desse conjunto de neuropeptídeos via 5-HT promove hiperpolarização de neurônios NPY/AgRP e despolarização de neurônios POMC/CART que resulta em efeitos anoréticos característicos como diminuição do consumo alimentar e perda de peso (HUSZAR, et al., 1997).

A nível periférico, o tecido adiposo interage com o SNC não somente do ponto de vista informativo, ou seja, sinalizando o atual estado energético do organismo, como também é um alvo direto das vias efetoras através do sistema nervoso simpático, induzindo uma resposta adaptativa que estimula tanto a lipólise como a termogênese. Nesse sentido, a depender das características e da localização, os adipócitos podem constituir tanto o tecido adiposo branco (TAB) como o tecido adiposo marrom (TAM), e influenciar diferentemente o metabolismo periférico dos triglicerídeos e o metabolismo energético em geral (BRITO, et al., 2007; BRUINSTROOP, et al., 2012; GEERLING, et al., 2014).

Enquanto o TAB é responsável por armazenamento de triglicerídeos e liberação de ácidos graxos livre e glicerol em processos catabólicos, o TAM utiliza os lipídeos para geração de energia térmica conhecida como termogênese (HALB e DA CUNHA, 2008). Em fases iniciais da vida, neonatos possuem uma quantidade relativamente maior de TAM que é responsável pela termogênese adaptativa nessa idade; uma estratégia de conservar o calor, uma vez que os mecanismos de termorregulação a nível central ainda não estão totalmente desenvolvidos (CANNON e NEDERGAARD, 2004). A progressão do seu desenvolvimento e função em roedores, ocorre entre a fase embrionária final e principalmente durante o período pós-natal até os 21 dias de vida, acompanhadas de uma involução após a lactação (CHABOWSKA-KITA e KOZAK, 2016). Muito embora sua presença em neonatos seja classicamente mais conhecida, a contribuição do TAM na termogênese de indivíduos adultos foi anteriormente confirmada através da captação de [18F]-fluorodeoxiglucose (FDG) por adipócitos marrons em regiões cervicais e supraclaviculares de pacientes oncológicos, em exames de tomografia computadorizada por emissão de pósitrons (PET) (NEDERGAARD, et al., 2007). Acredita-se que sua ativação nessa idade seja dependente de mecanismos de adaptação metabólica à estímulos como exposição ao frio e manipulação farmacológica e dietética (com aumento do consumo de glicose e ácidos graxos) (CHRISTENSEN, et al., 2006).

O TAM encontra-se em maior quantidade na região interescapular e em pequenos depósitos perirrenais e perivasculares, possuindo uma linhagem de desenvolvimento embrionário similar ao tecido muscular esquelético, que é mediado por células progenitoras que expressam o fator miogênico 5 ($Myf5^{+}$) (TOWNSEND e TSENG, 2012). Em contrapartida, células progenitoras Myf5 negativas são precursoras comuns do TAB e sob certos estímulos (como ativação simpática na termogênese induzida por dieta ou exposição ao frio) podem ser alvo de reguladores transcricionais para produção “de novo” de tecido adiposo “bege” ou tecido adiposo marrom “induzível”, ou para transdiferenciação de adipócitos maduros pré-existentes que adquirem propriedades termogênicas, processo conhecido como “browning” (FRONTINI e CINTI, 2010; TOWNSEND e TSENG, 2012).

Há evidências que sugerem a participação do sistema serotoninérgico central na ativação do TAM via tônus simpático, uma vez que a estimulação simpática do TAM na exposição ao frio pode ser mediada por serotonina (CANO, et al., 2003) e a ausência de neurônios serotoninérgicos ou a inibição de sua biossíntese central foi associada a ausência de termogênese no TAM, em camundongos (FULLER, et al., 1987; HODGES, et al., 2008). A interdependência também é notada por sinalização periférica uma vez que a leptina circulante por exemplo, que regula o consumo alimentar e o gasto energético, age no hipotálamo inibindo neurônios que expressam NPY, e no tecido adiposo marrom, favorecendo a termogênese (SALBE, et al., 1997; AMITANI, et al., 2013). Portanto, a assimilação desses estudos nos permite concluir que a serotonina central age de maneira integrada na regulação positiva do balanço energético; diminuindo o consumo alimentar, predominantemente por vias hipotalâmicas, e aumentando o gasto energético, por termogênese do TAM.

2.3 Mitocôndria e metabolismo energético

As mitocôndrias desempenham um papel biológico importante na manutenção da homeostase energética e, portanto, um bom funcionamento dessas organelas contribui para preservação da função celular. Sua descoberta, ainda no século XIX foi caracterizada pela identificação da sua estrutura interna e mais tarde, a constatação da sua ocorrência praticamente ubíqua no organismo (ERNSTER e SCHATZ, 1981). A função clássica das mitocôndrias definida por Mitchell a partir da teoria quimiosmótica da fosforilação oxidativa descreve a formação de energia associada a um gradiente eletroquímico através da membrana mitocondrial interna (MITCHELL, 1966). Entretanto, essa organela ainda participa de processos envolvidos no transporte de Ca^{2+} mitocondrial, por mediação de um canal uniporte que o direciona para a matriz mitocondrial conduzido por um potencial elétrico de membrana mitocondrial ($\Delta\Psi m$). Além disso, participa da produção de espécies reativas de oxigênio pela redução monovalente do oxigênio à água na cadeia respiratória, da sinalização REDOX, em especial pelo sistema das glutationas, e de mecanismos apoptóticos (KOOPMAN, et al., 2010).

Sua estrutura consiste em duas membranas lipoproteicas (bicamada lipídica) que delimitam seu formato alongado, são elas a membrana mitocondrial interna e externa, separadas por um espaço intermembranar. As dobras internas da membrana mitocondrial interna originam cristas que aumentam a superfície interna e entram em contato direto com a matriz mitocondrial, local das enzimas do ciclo do ácido cítrico e β-oxidação (NELSON e COX, 2008; KANG e PERVAIZ, 2012). Ligados à membrana interna, cinco complexos proteicos constituem a cadeia de transporte de elétrons envolvida na produção de energia acoplada à fosforilação oxidativa. Os substratos, portanto, são transportados para as células e passam por uma série de reações metabólicas até a formação de ATP, um intermediário rico em energia que é utilizado como a principal moeda energética celular através das mitocôndrias (HEPPLE, 2014). Para isso, o fluxo de elétrons na cadeia respiratória derivada de NADH via complexo I e $FADH_2$ via complexo II, ambos derivados do ciclo do ácido cítrico, é associado ao bombeamento de prótons pelos complexos I, III e IV através da membrana mitocondrial interna, que retornam à matriz mitocondrial pelo complexo V ressintetizando adenosina trifosfato (ATP) (SOUSA, et al., 2018).

Entretanto, nem toda a energia é armazenada como ATP. Por exemplo, no TAM a energia que é derivada dos combustíveis metabólicos é dissipada em um processo que é facilitado pelo escape de prótons, liberando assim o calor. A regulação do escape de prótons neste tecido é mediada pela proteína desacopladora mitocondrial (UCP) que está localizada na membrana mitocondrial interna (KRAUSS, et al., 2005). Desta forma, o TAM em especial desempenha um papel importante na regulação da termogênese e do balanço energético a partir da ativação de UCP 1, que encontra-se em abundância nesse tecido (CANNON e NEDERGAARD, 2004). A expressão de outras isoformas de UCP em diversos tecidos, como UCP 2 expressa de maneira ubíqua, UCP 3 predominantemente encontrada no tecido muscular estriado esquelético e cardíaco e UCP 4 e 5 mais expressas em regiões encefálicos (ZHAO, et al., 2019), parece estar mais associada com a regulação da produção de espécies reativas e de condições de estresse oxidativo (MAILLOUX e HARPER, 2011).

Decorrente de vias de sinalização que respondem a diferentes eventos estressores, algumas adaptações mitocondriais são bem conhecidas na

tentativa de auto-ajuste aos desafios bioenergéticos provenientes do ambiente. Essa adaptação corresponde à formação de novas organelas com uma sincrônica expressão gênica mediada pela mitocôndria e núcleo, onde aproximadamente 90% das proteínas mitocondriais são codificadas pelo genoma nuclear (PLOUMI, et al., 2017). Conduzidos por sensores energéticos como a proteína quinase dependente de adenosina monofosfato (AMPK), fatores de transcrição como o coativador 1 alfa do PPARγ, o PGC1α, induzem maior expressão ou atividade transcricional de proteínas críticas na biogênese mitocondrial. A exemplo, o fator de transcrição mitocondrial A (TFAM) e fatores respiratórios nucleares (NRF) 1 e 2 (JORNAYVAZ e SHULMAN, 2010). O PGC1α como um importante regulador da homeostase energética modula ainda sinalizações relacionadas à termogênese, quando associados a uma maior expressão da proteína domínio PR 16 (PRDM16), por exemplo, considerada um marcador específico do fenótipo marrom (TAM) envolvido no catabolismo de lipídeos e desacoplamento mitocondrial. Portanto, a promoção de biogênese mitocondrial e do fenômeno de “browning”, podem ser adquiridas de maneira adaptativa, sendo necessário a indução concomitante da expressão de UCP 1 para uma característica termogênica completa (GONZALEZ-GARCIA, et al., 2019).

2.4 Disfunção mitocondrial e estresse oxidativo na obesidade

O papel das mitocôndrias na obesidade está associado à integração de diferentes informações metabólicas como níveis de ATP, inflamação e estresse oxidativo, que comprometem a função mitocondrial e favorecem o desenvolvimento ou manutenção dessa condição clínica (LAHERA, et al., 2017). Como o principal sítio de formação das EROS são as mitocôndrias, o desequilíbrio oxidativo referente à sua função pode induzir o quadro de estresse oxidativo, onde o aumento da produção de EROS é superior à ativação dos sistemas de defesas antioxidantes mediados principalmente pela atividade de enzimas antioxidantes como superóxido dismutase (SOD), catalase e glutationa-S-transferase (GST) e da defesa não-enzimática, como o balanço REDOX mediado principalmente pelo sistema de glutationas (FERREIRA, et al., 2016). A

instalação de estresse oxidativo tem sido relacionado com dano celular e tecidual que compromete a estrutura e função de tecidos e desencadeia processos patológicos diversos (KOWALTOWSKI, et al., 2001)

Ainda decorrentes do desbalanço oxidativo, com a produção de EROS mitocondrial e o fluxo elevado de Ca^{2+} mitocondrial, a abertura do poro de transição de permeabilidade mitocondrial (PTPM) dissipa o gradiente eletroquímico prejudicando a produção de energia através da fosforilação oxidativa e por conseguinte, afeta o suprimento de energia celular (Vercesi et al., 1988; Gunter et al., 2004). Assim, ocorre o desdobramento da membrana mitocondrial interna conhecido como inchamento mitocondrial, que por sua vez pode romper a membrana mitocondrial externa e provocar extravasamento do conteúdo da matriz mitocondrial para o citosol, como proteínas pró-apoptóticas (citocromo c e Smac/DIABLO). Como consequência, a ativação de caspases e subsequente morte celular são fatores presentes em processos fisiopatológicos, que podem susceptibilizar o organismo ao desenvolvimento de diversas doenças crônicas na vida adulta (KOWALTOWSKI, et al., 2001; QUINTANILLA, et al., 2013).

Evidências derivadas de estudos clínicos relacionando obesidade e estresse oxidativo, estabeleceram uma correlação entre biomarcadores de estresse oxidativo (tais como peroxidação lipídica e oxidação proteica) com o índice de massa corporal (IMC) elevado (VINCENT e TAYLOR, 2006; SANKHLA, et al., 2012). Células adiposas aumentadas são significantes fontes de produção de EROS (indutores de estresse oxidativo), como já é documentado na literatura (HOUSTIS, et al., 2006). Além disso, o excesso de calorias característico da obesidade sobrecarrega o ciclo de Krebs e a cadeia respiratória mitocondrial, favorecendo o quadro de estresse oxidativo e alterando a função de diferentes tipos celulares, sendo um importante fator na indução de doenças metabólicas relacionadas à obesidade (MANNA e JAIN, 2015; TAN, et al., 2018).

2.5 Manipulação serotoninérgica e modulação da bioenergética mitocondrial e balanço oxidativo

Ainda não há um consenso sobre a interação entre o sistema serotoninérgico e as mitocôndrias. Em termos de processamento metabólico, a monoamina oxidase (MAO) representa um elo importante entre serotonina (5-HT) e mitocôndrias. Essa flavoenzima liga-se à membrana mitocondrial externa e é responsável pela desaminação de monoaminas, como a 5-HT, e catecolaminas, regulando a liberação de vesículas sinápticas serotoninérgicas com produção de amônia e peróxido de hidrogênio (SANDLER e YOUDIM, 1972).

Além disso, também tem sido relatado o envolvimento da 5-HT na indução de biogênese mitocondrial. Rasbach et al. 2009, por exemplo, demonstrou esse efeito com agonista de receptores 5-HT2 no tecido renal (RASBACH, et al., 2010). Além disso, há relatos de aumento de biogênese mitocondrial no córtex renal, fígado e coração através de agonista de receptores 5-HT1F e 5-HT2A (GARRETT, et al., 2014; HARMON, et al., 2016). No SNC, a participação do receptor 5-HT2A mostrou-se eficaz na indução de biogênese mitocondrial em neurônios corticais de camundongos via sinalização de SIRT1-PGC1α (FANIBUNDA, et al., 2019). Já no estriado e substância nigra, o tratamento com agonista de receptor 5-HT1F induziu biogênese mitocondrial em um modelo de camundongos para doença de Parkinson (SCHOLPA, et al., 2018). Diante das evidências, fica claro que o 5-HT desempenha uma contribuição diversificada nas funções biológicas, embora o entendimento de sua interação com as mitocôndrias ainda precise de mais investigações uma vez que a maioria dos estudos utilizam exposição farmacológica com agonistas de receptores serotoninérgicos ou infusão direta de 5-HT de maneira aguda e *in vitro*.

Há evidências sobre a modulação da produção de EROS mitocondrial a partir das UCPs mediada por serotonina (BRAND, et al., 2002; FANG, et al., 2013) e portanto sua influência sobre a regulação do balanço oxidativo. Um desajuste nessa regulação pode induzir quadros de estresse oxidativo em macromoléculas e estruturas celulares, situação que em último caso poderia resultar em morte celular (SIES e MEHLHORN, 1986; GUTTERIDGE, 1993). Nesse contexto, diversos estudos têm sugerido o envolvimento da disfunção mitocondrial no aparecimento de doenças neurodegenerativas (CENINI, et al.,

2019), bem como na obesidade (DE MELLO, et al., 2018), diabetes (WADA e NAKATSUKA, 2016) e doenças cardiovasculares (CHISTIAKOV, et al., 2018).

Sobre a relação entre concentrações de serotonina e estresse oxidativo, estudos já demonstraram que fármacos que modulam o sistema serotoninérgico, como a Fluoxetina (Flx) ou Prozac, um inibidor seletivo de recaptação de serotonina (ISRS), demonstram capacidade de reduzir os níveis de EROS, possibilitando um benefício antioxidante (KHANZODE, et al., 2003; ZAFIR, et al., 2009; AHMAD, et al., 2010; MORETTI, et al., 2012). Essa classe de fármacos possui grande relevância e preferência no tratamento de transtornos depressivos e de ansiedade pois possuem especificidade de atuação, reduzidos efeitos colaterais e boa tolerabilidade, e seu mecanismo de ação consiste no bloqueio do transportador de serotonina (SERT) no neurônio pré-sináptico e aumento da concentração de 5-HT na fenda sináptica e da sua neurotransmissão (MASAND e GUPTA, 1999; KRISHNAN e NESTLER, 2008).

A conversão metabólica da fluoxetina à norfluoxetina é realizada no fígado, e esse metabólito ativo possui características peculiares e bem descritas na literatura, como um longo tempo de meia-vida, facilidade em ultrapassar a placenta e a barreira hematoencefálica (Francis-Oliveira et al., 2013), além de ser encontrada também no leite materno (Davanzo et al., 2011). Entre outros antidepressivos, a fluoxetina é o fármaco identificado em maior nível em neonatos cujas mães seguiram o tratamento farmacológico em questão (Weissman et al., 2004).

Embora ainda não totalmente elucidada, a relação entre fluoxetina e mitocôndrias é estudada há um certo tempo. Já é conhecido que a Flx penetra nas membranas plasmáticas e pode ser detectado nas mitocôndrias, sinaptossomas e outras organelas celulares (Oliveira, 2016). Após aplicação oral ou intravenosa em ratos, 40% do fármaco demonstrou estar presente em mitocôndrias e sinaptossomas, tornando essas frações subcelulares um possível local de ação de drogas (Moretti et al., 2003). Estudos recentes do nosso grupo de pesquisa demonstraram que o tratamento crônico com fluoxetina durante a lactação modula positivamente a bioenergética mitocondrial e balanço oxidativo nos tecidos controladores do metabolismo energético (hipotálamo, TAM e

músculo esquelético) em ratos machos aos 60 dias de vida, o que suporta a proposição de que o sistema serotoninérgico atua na regulação do aporte e dispêndio energético (DA SILVA, et al., 2015; DA SILVA, et al., 2015). A partir dessas evidências, constatamos que o potencial modulatório da serotonina em mecanismos associados à melhora do balanço energético do ponto de vista mitocondrial representa uma lacuna na literatura científica. Dessa maneira, esse estudo pode contribuir para uma maior compreensão integrativa dessa temática, associada ao sobrepeso/obesidade.

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar, em ratos *Wistar* machos, submetidos ou não à supernutrição durante a lactação e à administração crônica de fluoxetina, a bioenergética mitocondrial, balanço oxidativo e expressão de moduladores do balanço energético corporal no hipotálamo e tecidos adiposos branco e marrom.

3.2 Objetivos Específicos

Avaliar, aos 60 dias de vida, em ratos *Wistar* machos normonutridos e supernutridos, submetidos ou não à administração crônica de fluoxetina:

3.2.1 O peso corporal durante a lactação e a manipulação farmacológica;

3.2.2 O consumo de oxigênio e a produção de espécies reativas em mitocôndrias isoladas do hipotálamo e tecido adiposo marrom;

3.2.3 Os biomarcadores de estresse oxidativo (MDA e Carbonilas), e a concentração de GSH e GSSG bem como o balanço REDOX no hipotálamo e tecido adiposo marrom;

3.2.4 A expressão proteica de UCP1 no tecido adiposo marrom;

3.2.5 A expressão gênica de *Ucp2, Ampk, Pgc1α, Tfam, Ndufb8, Sdhb e Atp5a* no hipotálamo;

3.2.6 A expressão gênica de *Ucp1* e *leptina* nos tecidos adiposos branco e marrom e de *Sirt1, Pgc1α e Prdm16* no tecido adiposo branco.

4. MATERIAIS E MÉTODOS

4.1 Animais e modelo de supernutrição pós-natal

Foram utilizadas ratas da linhagem *Wistar* com 80 dias de vida provenientes da colônia do Departamento de Nutrição da Universidade Federal de Pernambuco. As fêmeas selecionadas, entre 150-200g, foram abrigadas em biotério com ciclo claro-escuro invertido (12/12 horas) sob condições padrão de temperatura, iluminação e umidade, com água e comida (dieta comercial – Presence, 74,5% de carboidratos, 23% de proteínas e 2,5% de lipídeos) *ad libitum*. As fêmeas foram aclimatadas em biotério durante 15 dias para sincronização do ciclo circadiano. Após a adaptação, as ratas, quando em período pró-estral, foram acasaladas na proporção de 2 fêmeas para 1 macho (200-250g) e após a detecção da prenhez foram transferidas para gaiolas individuais. Após o nascimento dos neonatos, os machos foram divididos aleatoriamente (com peso de 6-8g) em dois grupos experimentais: Grupo Normonutrido (n=9 por lactante) e Grupo Supernutrido (n=3 por lactante) conforme previamente publicado (PLAGEMANN, et al., 1992; PLAGEMANN, 2011; PLAGEMANN, et al., 2012). Aos 21 dias de vida, os animais foram desmamados e separados em gaiolas de acordo com o grupo experimental e passaram a receber água e ração (dieta comercial – Presence) livres até a idade experimental (60 dias de vida). Todos os procedimentos éticos da utilização de animais para pesquisa científica seguiram as recomendações do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e o presente estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Pernambuco (processo de nº 0027/2017 - Anexo A).

4.2 Manipulação farmacológica e grupos experimentais

Aos 39 dias de vida, os grupos foram subdivididos conforme a manipulação farmacológica: Grupo Normonutrido + Veículo (solução salina – NaCl 0,9%), Grupo Normonutrido + Fluoxetina (fluoxetina - 10mg/kg de peso corporal), Grupo Supernutrido + Veículo, e Grupo Supernutrido + Fluoxetina. Todos os grupos

foram tratados diariamente por via subcutânea do 39º ao 59º dia de vida durante a segunda hora após início do ciclo escuro (DA SILVA, et al., 2019).

4.3 Avaliação do peso corporal e Índice de Lee

O peso corporal dos animais de ambos os grupos experimentais foi mensurado durante o período de lactação (3º, 7º, 14º e 21º dia de vida), aos 30 dias de vida e também durante o tratamento farmacológico (39º ao 59º dia de vida). O peso foi registrado no início do ciclo escuro através de balança eletrônica digital (Marte, modelo S-100 com sensibilidade de 1g). O Índice de Lee foi mensurado, aos 60 dias de vida, através da razão entre a raíz cúbica do peso corporal (g) e o comprimento naso-anal (cm) multiplicado por 1000 (BERNARDIS e PATTERSON, 1968).

4.4 Sacrifício dos animais e coleta dos materiais biológicos

Aos 60 dias de vida, os animais foram eutanasiados por decapitação em guilhotina e os tecidos rapidamente coletados e acondicionados em gelo temporariamente durante a coleta. O hipotálamo foi dissecado com pinça cirúrgica através de incisões bilaterais no sentido longitudinal e retirado em aprofundamento de aproximadamente 2mm. O tecido adiposo marrom foi extraído da região interescapular e o tecido adiposo branco da região epididimal e retroperitoneal, cuidadosamente dissecados para remoção de quaisquer outros resíduos teciduais. Após a coleta, as amostras foram tratadas de acordo com as análises subsequentes (isolamento mitocondrial, quantificação de biomarcadores, PCR ou *Western Blotting*) e armazenadas em freezer -20ºC

4.5 Isolamento mitocondrial

Após a coleta, os tecidos foram imediatamente homogeneizados (IKA® 150 RW 20, Germany) em tampão de isolamento contendo Manitol 225mM, Sacarose 75mM, EGTA 1mM e HEPES 4mM, pH 7,2 e centrifugadas diferencialmente para obtenção das mitocôndrias: Inicialmente à 1180xg por

10min a 4ºC para retirada do sobrenadante, e este por sua vez submetido a uma segunda centrifugação de 12000xg durante 10min a 4ºC. O pellet (conteúdo mitocondrial) foi então ressuspendido em tampão específico (Sacarose 250 mM e HEPES 5mM, pH 7,2) (LAGRANHA, et al., 2010; DA SILVA, et al., 2015). As mitocôndrias coletadas foram submetidas à quantificação de proteínas totais pelo método de Bradfrod utilizando proteína do soro bovino (BSA) como padrão (BRADFORD, 1976). A leitura foi realizada em espectrofotômetro (Even, modelo IL-512, UV/VIS) a 495nm.

4.6 Avaliação do consumo de oxigênio e espécies reativas mitocondriais

O consumo de oxigênio em suspensões de mitocôndrias isoladas foi realizado polarograficamente utilizando-se um eletrodo tipo Clark (Hansatech Instruments, Pentney King's Lynn, UK). As suspensões foram mantidas em câmara de vidro fechada, termostatizada a 28°C e sob agitação constante, em meio de reação contendo KCl 120 mM, HEPES 4 mM, KH_2PO_4 5 mM, EGTA 1 mM e BSA 0,2%, pH 7,2. A respiração mitocondrial foi mensurada utilizando 0,5mg de proteína/ml de meio de reação, substratos do complexo I (glutamato 10mM e malato 2mM) e do complexo II (succinato 5mM + rotenona 4µM). Para as mitocôndrias de hipotálamo a avaliação do consumo de oxigênio deu-se através da adição de ADP 0,5mM, Oligomicina 1µg/mL e CCCP 1µM. Para a análise no tecido adiposo marrom utilizou-se substratos do complexo II (succinato 5mM + rotenona 4µM), GDP (inibidor de UCP 1 – 1mM) e CCCP 1µM (DA SILVA, et al., 2015).

A produção de espécies reativas (oxigênio e nitrogênio) em mitocôndrias isoladas (0,1mg de proteína/ml do meio de reação) foi avaliada com substratos do complexo II (succinato 5mM + rotenona 4µM) para ambos os tecidos e monitorada por técnica fluorimétrica, usando o corante permeável a membrana H_2DCF-DA (diacetato de diclorodihidrofluorenceína, 5 µM) em 485 nm de excitação e 530 nm de emissão no leitor de fluorescência FLUOStart (OMEGA, USA) (DA SILVA, et al., 2015).

4.7 Processamento dos tecidos para análises do balanço oxidativo

Para as análises do balanço oxidativo o hipotálamo e tecido adiposo marrom foram homogeneizados em tampão de extração (Tris base 100 mM, pH 7,5; EDTA 10 mM; Nonidet 1%, PMSF 2mM e Ortovonadato 1 mM). Para quantificação proteica e demais análises, as amostras foram centrifugadas (Sigma, model1-14K, Germany) a 1180xg, a 4°C, por 10 minutos e somente o sobrenadante foi utilizado (DA SILVA, et al., 2015; BRAZ, et al., 2016). O conteúdo de proteína total no homogeinato do hipotálamo e tecido adiposo marrom foi mensurado pelo método de Bradford (BRADFORD, 1976) e para as análises subsequentes utilizou-se a concentração de 0,3mg/ml do sobrenadante.

4.8 Avaliação da peroxidação lipídica

Para mensurar o nível de peroxidação lipídica utilizou-se a técnica colorimétrica de Buege e Aust (BUEGE e AUST, 1978), onde uma alíquota do sobrenadante (0,3mg/ml) foi incubada com ácido tricloroacético (30%) e Tris HCl (10mM, pH 7,4) e centrifugados a 1180xg por 10min. Em seguida, ao sobrenadante adicionou-se ácido tiobarbitúrico (TBA) 0,73% em igual volume para posterior incubação a 100ºC durante 15min. O produto dessa reação, o malondialdeído (MDA), é uma das substâncias reativas ao ácido tiobarbitúrico (TBARS) que pode apresentar coloração rosada, sendo mensurada em espectrofotômetro (Even, modelo IL-512, UV/VIS) a 535nm. Os resultados de MDA foram expressos em mmol/mg de proteína.

4.9 Avaliação da oxidação de proteínas

O conteúdo de carbonilas foi mensurado de acordo com a metodologia descrita por Levine (LEVINE, et al., 1990) para avaliar o nível de oxidação proteica nas amostras. Inicialmente foi adicionado às amostras (0,3mg/ml) ácido tricloroacético (30%) e a mistura foi mantida em gelo durante 15min. Em seguida, centrifugou-se a 1180xg por 15min. Ao pellet adicionou-se 2,4-dinitrofenilhidrazina (DNPH) a 10mM com posterior incubação no escuro durante

1h. O pellet então foi submetido a um processo de três lavagens com etanol/acetato de etila (1:1) intercalados por centrifugação a 1180xg por 5min. Ao final, o pellet foi incubado com guanidina 6M a 37ºC, por 30 min e a absorbância mensurada a 370nm. Os resultados foram expressos em nmol de carbonilas/mg de proteína.

4.10 Avaliação do estado REDOX

Para quantificação da concentração de glutationa reduzida (GSH), adicionou-se às amostras (0,3mg/ml) tampão fosfato 0,1M (pH 8,0) + EDTA 5mM. A mensuração do conteúdo de glutationa oxidada (GSSG) deu-se através da incubação das amostras com N-ethylmaleimide (NEM) 0,04M, seguida da adição de Tampão Hidróxido de Sódio (NaOH) 0,1M. Para ambos os ensaios, as amostras foram incubadas com o-phthaldialdehyde (OPT) 1mg/mL durante 15min em temperatura ambiente. A intensidade de fluorescência foi realizada em espectrofluorímetro (FLUOStart, OMEGA, USA) nos comprimentos de onda de 350nm de excitação e 420nm de emissão. Para comparação dos valores, foi realizada uma curva com concentrações padrões conhecidas de GSH e GSSG. Os valores foram apresentados em µmol/mg de proteína e o balanço REDOX representado pela razão entre os valores de GSH e GSSG (HISSIN e HILF, 1976).

4.11 Mensuração do índice oxidativo global (Oxy-score)

A estimativa do dano oxidativo global nos tecidos analisados foi realizada a partir da diferença entre o somatório dos valores padronizados dos biomarcadores antioxidantes (GSH) e oxidantes (MDA, Carbonilas, GSSG) analisados, seguindo a seguinte equação: OXY-SCORE= (ANTIOXI – OXY). Dessa maneira, valores positivos indicam uma prevalência de capacidade antioxidante e valores negativos sugerem uma propensão a um dano oxidativo. Os dados foram expressos em unidades arbitrárias (RODRIGUEZ-RODRIGUEZ, et al., 2015).

4.12 Avaliação da expressão gênica por PCR em tempo real

Inicialmente o RNA total foi extraído pelo método de extração com isotiocinato de guanidina e o reagente Trizol (Invitrogen, USA) na razão 1:1,5 de amostra (CHOMCZYNSKI e SACCHI, 1987). Após a homogeneização dos tecidos (IKA® 150 RW 20, Germany), houve uma incubação de 5 minutos à temperatura ambiente e posterior acréscimo de clorofórmio estabilizado (P.A) para centrifugação a 12000xg, a 4ºC por 15 minutos. Ao RNA formado, adicionou-se álcool isopropílico (P.A.) e acetato de amônia 2M e a mistura foi então incubada a -20ºC por 20 min, seguido de uma nova centrifugação a 12000xg, a 4ºC por 10 minutos. O pellet (RNA) foi então lavado em etanol a 75% e centrifugado a 7500xg, 4ºC por 5 minutos. O sedimento de RNA foi ressuspenso em água livre de RNase e armazenado em -20ºC durante os experimentos (CIRERA, 2013; DA SILVA, et al., 2019). Para a quantificação do RNA, utilizou-se o Nanodrop 2000 (Thermo Scientific, USA) onde o grau de pureza de todas as amostras foi superior a 1.8 admitindo-se a razão entre os valores de absorbância a 260nm e 280nm (LAGRANHA, et al., 2007).

Para a análise de expressão gênica, utilizou-se a técnica da reação em cadeia da polimerase (PCR) em tempo real através do Rotor-Gene Q (Qiagen, USA). As amostras foram utilizadas na concentração de 700ng (analisadas em duplicata) e os parâmetros utilizados em cada reação seguiram as normas do fabricante (SuperScript III Platinum SYBR Green One-Step qRT-PCR kit – Invitrogen, USA). Os seguintes genes foram avaliados:

Quadro 1 – Genes analisados no hipotálamo, tecido adiposo marrom e tecido adiposo branco de ratos machos *Wistar* aos 60 dias de vida

	Gene	**Sigla**
HIPOTÁLAMO	Beta-2-microglobulina – controle interno (β-2-microglobulin)	*β-2M*
	Proteína desacopladora mitocondrial 2 (Uncoupling protein 2)	*Ucp2*
	Proteína kinase dependente de AMP	*Ampk*

	(AMP-activated protein kinase)	
	Coativador 1 de PPAR γ (Peroxisome activated receptor gamma coactivator 1 alpha)	*Pgc1-α*
	Fator respiratório nuclear 2 (Nuclear respiratory factor 2)	*Nrf2*
	Subunidade B8 do complexo mitocondrial I (NADH:Ubiquinone Oxidoreductase Subunit B8)	*Ndufb8*
	Subunidade B do complexo mitocondrial II (Succinate Dehydrogenase Complex Iron Sulfur Subunit B)	*Sdhb*
	Subunidade alfa do complexo mitocondrial V (ATP synthase alpha-subunit)	*Atp5a*
TAM	Beta-actina – controle interno (β-actin)	-
	Proteína desacopladora mitocondrial 1 (Uncoupling protein 1)	*Ucp1*
	Leptina (Leptin)	-
TABr	Beta-2-microglobulina – controle interno (β-2-microglobulin)	*β-2M*
	Leptina (Leptin)	-
TABe	Beta-2-microglobulina – controle interno (β-2-microglobulin)	*β-2M*
	Sirtuína 1 (Sirtuin 1)	*Sirt 1*
	Coativador 1 alfa do PPAR γ (Peroxisome activated receptor gamma coactivator 1 alpha)	*Pgc1*
	Proteína domínio PR 16 (PR/SET domain 16)	*Prdm16*
	Proteína desacopladora mitocondrial 1 (Uncoupling protein 1)	*Ucp1*

Legenda: TAM – Tecido Adiposo Marrom; TABr – Tecido Adiposo Branco Retroperitoneal; TABe – Tecido Adiposo Branco Epididimal. Fonte: O Autor, 2020.

As especificações dos primers utilizados encontram-se nos artigos do hipotálamo (Tabela 1 – Artigo 2) e do tecido adiposo marrom (Tabela 1 – Artigo 3). Após a comparação dos valores do CT (Cycle Threshold) de cada gene de interesse com o respectivo gene normalizador para cada tecido, a expressão relativa de RNA mensageiro foi expressa de acordo com o cálculo de $2^{-\Delta\Delta Ct}$ (LIVAK e SCHMITTGEN, 2001).

4.13 Avaliação da expressão proteica por *Western Blotting*

Alíquotas das mitocôndrias de tecido adiposo marrom foram utilizadas para mensuração do conteúdo de proteínas totais (BRADFORD, 1976). Primeiramente as amostras (30µg/ml) foram submetidas a uma eletroforese em gel de SDS-poliacrilamida a 12% por 90 minutos a 100V em tampão de eletroforese Tris-Glicina (Tris 250mM, Glicina 192 mM, pH 8,3-9,3) (Towbin 1979). As proteínas do gel foram transferidas para membrana de PVDF (polyvinylidene fluoride), previamente ativadas em álcool metílico P.A., a 15V, *overnight.* A eficiência da transferência foi verificada usando o corante reversível Ponceau-S (LAGRANHA, et al., 2010). Após a transferência, as ligações não específicas foram bloqueadas pela incubação das membranas com solução TBS-Tween (TBS-T) contendo leite desnatado 10% (TBS 1x, Tween 20 0,1%) por 60 minutos. Após o bloqueio, as membranas foram lavadas 3 vezes em TBS-T, sob agitação, por 5 minutos e incubadas a 4ºC com anticorpo primário para UCP1 (SCBT: SC-6528), em solução TBS-T contendo leite desnatado, *overnight.* Após nova sequência de lavagens, foram incubadas com anticorpo secundário anti-goat IgG conjugada a enzima HRP (SCBT: SC2768) em solução tampão, a 25ºC, por 4h. Posteriormente, as membranas foram lavadas e incubadas com substrato para HRP e intensificador de quimioluminescência (ECL Western Blotting System) por 5 min para exposição imediata a filme de raio-X e processamento de maneira convencional (câmara escura e solução reveladora e fixadora de raio X). A intensidade das bandas do Western Blotting foram analisadas e quantificadas por densitometria óptica usando o software Image J (NIH, Maryland, USA) e a representação dos dados feita em unidades arbitrárias (DA SILVA, et al., 2015; FERREIRA, et al., 2019).

4.14 Analise estatística

Todos os dados foram analisados segundo a normalidade de distribuição pelo teste Kolmogorov-Smirnov e encontraram-se dentro da distribuição gaussiana sendo posteriormente analisados pelo teste ANOVA two-way com comparações múltiplas de Tukey. O nível de significância adoto foi mantido em 5% para todas as análises e os dados foram expressos em média ± erro padrão da média. A construção do banco de dados e as análises estatísticas foram desenvolvidas no programa Excel (Microsoft, USA, versão 2016) e Graphpad Prism 6.0 (GraphPad Software Inc., La Jolla, Ca, USA) respectivamente.

5 RESULTADOS

5.1 Artigo 1 - Systematic review of serotonin reuptake inhibitors (SSRI) effects on the rat brain mitochondria

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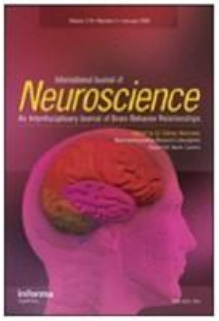

Systematic review of serotonin reuptake inhibitors (SSRI) effects on the rat brain mitochondria

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Systematic review of serotonin reuptake inhibitors (SSRI) effects on the rat brain mitochondria

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Running title: Mitochondrial SSRI effects on rat brain diseases

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Abstract

Serotonin reuptake inhibitors (SSRI) are typically selected for the treatment of depression due to their safety, efficacy and minimal side effects. Currently, the use of SSRI in other disorders that would not usually receive such treatment has shown intriguing outcomes. Although little is known about SSRI interactions in these conditions, neurodegenerative and metabolic impairments have been demonstrated to be responsive to antidepressant treatment. Given that intracellular signaling pathways rely on environmental stimuli and intrinsic homeostatic patterns, mitochondria adjustments to drug exposition can result in cellular bioenergetic modulation, although how mitochondria change their function in response to pharmacological stimuli is not completely clear. Thus, the present review aimed to overview the interaction between SSRIs and mitochondrial function in experimental rat models of brain disease. Using pre-defined protocols that were registered on the PROSPERO website, database searches were conducted in Medline/PubMed, ScienceDirect, Scopus and Web of Science. A total of 317 studies were found, and 5 studies were included in this systematic review. Furthermore, the PRISMA statement was used for reporting this systematic review, guiding the problem construction and exploring the correlation between SSRI use and mitochondrial effects. The majority of studies demonstrated that SSRI exposure in brain dysfunction models improved mitochondrial metabolism, antioxidant enzymes activity and energy production. The findings suggest that SSRI exposure in neuropathology conditions may positively modulate mitochondrial structure and function in several brain regions, indicating a pivotal interaction of this organelle with the serotonin system.

Keywords: mitochondria; brain disease; serotonin reuptake inhibitors

1. Introduction

Growing evidence has demonstrated a broad spectrum of action of selective serotonin reuptake inhibitors (SSRI) in psychologic conditions as depression. Antagonizing serotonin transporters (SERT), drugs belonging to this class can increase serotonin availability (5-HT) in the synaptic cleft and, hence, its neurotransmission (Masand et al., 1999). As first-line drugs in the treatment of psychological and anxiety disorders, SSRI modulate the serotonergic system, and their presence can be detected in several brain structures and tissues due to their strong diffusion capacity; SSRI can cross the blood-brain barrier and have been found in breastmilk and the placenta, in cases of pregnant women treated with these drugs (Francis-Oliveira et al., 2013; Davanzo et al., 2011). Therefore, the complexity of SSRI highlights the importance of better understanding how these agents interact in the cellular environment.

All biological processes are dependent on the maintenance of cellular energetic homeostasis. In this sense, the efficient functioning of the mitochondria may certainly preserve the integrity of the intracellular process. In fact, the well-established, classic function of mitochondria as defined by Mitchell is that of ATP synthesis, which is accomplished by an electrochemical gradient across the inner mitochondrial membrane (Mitchell, 1966); however, the mitochondria is also involved in calcium flow, reactive oxygen species (ROS) production, redox signaling and apoptotic mechanisms (Koopman et al., 2010). Thus, interactions of mitochondria according to the environmental stimuli may affect its function and differently modulate its adaptive responses.

Most antidepressants can accumulate in the brain, membranes and subcellular

components (Hroudová et al., 2012), and this higher concentration is an interesting feature to study that can clarify these drugs' effects on cellular bioenergetics. In this regard, intracellular signaling pathways are dependent on ATP storage (Villa et al., 2017), and disturbances in these conditions can result in metabolic consequences for cells. As a matter of fact, although SSRI drugs are known for their clinical safety and minimal side effects (Krishnan et al., 2008), their mitochondrial interactions have already been demonstrated as in Moretti et al.'s study that reported the presence of 40% of fluoxetine in the mitochondria and synaptosomes of rats treated with this SSRI (Moretti et al., 2003). As many factors, such as the drug nature, dosage, route of administration and total time of exposure, can alter mitochondrial adjustments, experimental studies support such concerns and complement primary knowledge in regard to prospective therapeutic actions. Therefore, the present review was performed to overview the interaction between SSRIs and mitochondrial function in experimental rat models of brain diseases.

2. Materials and Methods

2.1 Search strategy

This systematic review was prospectively registered with the PROSPERO database (CRD42018104826) and was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) standard (Moher et al., 2009). Two independent authors (GRFB and AIS) searched PubMed, ScienceDirect, Scopus and the Web of Science database in July 2019 for controlled experimental studies. The following MeSH terms were used: "serotonin reuptake inhibitors" OR

"SSRI", "mitochondria" and "brain" in the title, abstract or index term fields. The authors applied the eligibility criteria according to the PICOS strategy: Population – rats; Intervention – SSRI administration/treatment; Comparison – control group (treated with vehicle solution); Outcomes – mitochondrial measurements; Study type – experimental studies. After the removal of duplicates, reviewers screened the titles and abstracts of all potentially eligible articles. The reviewers then considered the full texts of these articles, and the final list of included articles was reached through consensus. The third reviewer (CJL) was available for mediation throughout the entire process; however, mediation was not required due to the consistent agreement between the authors.

2.2 Inclusion criteria

We included studies conducted with SSRI administration in rats with brain dysfunction/injury, studies that reported mitochondrial outcomes, studies conducted with the SSRI class of antidepressants and studies that reported use of a control group. In addition, book chapters, meeting abstracts and annals events were excluded. There were no language restrictions or restrictions by year of publication.

2.3 Data extraction

Regarding experimental models, the authors extracted the author and year of each study, as well as the sex, species, age/weight from the experimental groups, the SSRI used, dosage, route of administration, duration of administration/treatment and brain region analyzed. Additionally, we extracted the aim of each study, reporting their

main outcomes and conclusions. Primary outcomes mainly included the activity of mitochondrial complexes, mitochondrial energy production and protein expression by proteomics.

2.4 Quality assessment

Animal studies included were assessed for risk of bias by SIRCLE's tool (Hooijmans et al., 2014), and the Kappa index was calculated to evaluate the inter-reviewer agreement (Kappa = 1 for all parameters). The following items were evaluated: sequence generation, baseline characteristics, allocation concealment, housing conditions, blinding of investigators, random outcome assessment, blinding of outcomes assessors, incomplete outcome data, selective outcome reporting, and other biases (Table 1).

3. Results and Discussion

3.1 Search results and flow of trials through the review

A total of 317 records were identified, which was reduced to 110 after the duplicates were removed. After screening and application of the eligibility criteria, 5 eligible studies were identified and included in our review. Figure 1 shows the working flow of our studies through the review.

3.2 Characteristics of the included studies

Table 2 summarizes the main experimental and pharmacological characteristics of the included studies. Five studies included adult male rat models with a body weight ranging from 200 g to 400 g. The SSRI most frequently studied in the included studies was fluoxetine followed by sertraline, which are two of the most commonly sold and recommended SSRI for depression treatment (Hansen et al., 2017). The studies were conducted in vivo and focused on SSRI mitochondrial effects in brain structures as discussed below. Among the included studies, the hippocampus was the main structure studied, but the cortex, striatum and raphe nuclei also received attention.

Table 3 reports the main mitochondrial analyses and outcomes from the included studies. Two studies included mitochondrial complex activity among their analyses (Kumar et al., 2010; Kumar et al., 2009), one study analyzed the mitochondrial energy production specifically (Wen et al., 2014) and two studies evaluated mitochondrial protein expression (Glombik et al., 2017; Peric et al., 2018). The majority of studies reported improvement/restoration of mitochondrial function after SSRI exposure.

The present review focused on the mitochondrial effects of SSRI exposure in brain regions given that the central nervous system is the intended target of antidepressants; however, in terms of bioenergetics, there is little information available concerning the effects of SSRI. Importantly, the present review reported only animal models of SSRI administration for brain diseases to reveal the possible therapeutic action of this class of antidepressants related to mitochondrial structure and function in disease processes.

The examination of SSRI in these studies contributes to understanding of the possible mechanisms of neuroprotection, and these studies can certainly help in the

understanding of the effects of antidepressants in neurodegenerative processes, such as in Huntington's disease (Kumar et al., 2009). In this study, sertraline (5 mg/kg b.w. and 10 mg/kg b.w.) effectively rescued the effects of neurotoxin 3-Nitropropionic acid (3-NP), a mitochondrial toxin that inhibits mitochondrial complex II activity irreversibly (Brouillet et al., 2005). Thus, the mitochondrial complexes I, II, III and IV activities were improved in the cortex, striatum and hippocampus. In a second study, the authors reinforced these data by showing a concentration-dependent effect of sertraline on the mitochondrial complex enzyme in animals with impaired hippocampal function by 3-NP neurotoxicity induction (Kumar et al., 2010). Important to mention, mitochondrial complexes activity impairment can lead to a process known as mitochondrial permeability transition pore (MPTP), where the calcium overload, membrane potential disruption and excessive ROS production may induce mitochondrial swelling and non-selective voltage-dependent mitochondrial channel opening (Rasheed et al., 2017). Inducement of pro-apoptotic mechanisms could then be one of the main mechanisms related to mitochondrial dysfunction that induce several neurodegenerative disorders (Quintanilla et al., 2017). The ability of antidepressants to strongly inhibit the mitochondrial permeability transition pore and the extracellular catabolism of ATP is one of the plausible explanations for improvements in mitochondrial respiratory complex function (Zhang et al., 2008). In addition, a neurodegenerative process that is naturally promoted by oxidative stress was reduced by an improvement in cellular excitotoxicity. In fact, the effects of antidepressants on neuronal protection, particularly in Huntington's disease, has been discussed in regard to their roles in modulating signaling pathways and contributing to the improvement of neurodegenerative disorders with mitochondrial abnormalities (Jamwal et al., 2015).

The mechanisms of action proposed by these studies could perhaps be further investigated. These mechanisms are orientated towards modifications of increased neuronal number and function due to increased 5-HT extracellular levels, inhibited voltage dependent channels and, hence, decreased cellular excitotoxicity and altered the signaling pathways of monoaminergic drugs, such as the increased expression of cyclic AMP response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF). Together, these mechanisms contribute to decreased mitochondrial ROS signaling and oxidative stress.

Studies concerning mitochondrial activity in response to antidepressants have shown interesting results, as evidenced by Wen et al., 2014; in that study, depressed rats showed an increased mitochondrial respiratory control rate (RCR) and ATP synthesis, and fluoxetine (5 mg/kg b.w.) decreased this rate in the raphe nuclei (Wen et al., 2014). As a measure to indicate the coupling between respiration and phosphorylation, RCR represents a quality control data of isolated mitochondria. Due to metabolic and mitochondrial dysfunction usually found in depression models, the results found by Wen et al., 2014 (increased respiratory control rate and ATP synthesis) did not correspond to the majority of the research in this field. The mitochondrial activity demonstrated in depressed rats had an opposite response after fluoxetine treatment, and by the authors' point of view, this response may represent a compensatory mechanism of increased cellular oxidative stress. Differences may also be related to the route of administration (intragastrically) and drug concentration (5 mg/kg b.w.), since drug treatment variables interfere in the final response. Altered energy metabolism due to depression-like disease is an usual disturbance that occurs as a disturbance in mitochondrial function (Bansal et al., 2016; Villa et al., 2018) and indeed proposed as a

mechanism of depression onset (mitochondrial hypothesis of depression) (Klinedinst et al., 2015). With that said, the results found by Wen and colleagues does not agree with the majority of studies in this research field and need to be explored deeply.

Metabolic impairments are common characteristics in the pathophysiology process of many diseases, and modulation of energy metabolism by antidepressants could be an important mechanism of action of these drugs. Therefore, proteomic analyses are increasingly being included in cutting edge research. Glombik et al., 2017 described interesting results using fluoxetine (10 mg/kg) as the SSRI of choice and reported upregulation of several mitochondrial proteins that regulate oxidative metabolism and mitochondrial biogenesis (Glombik et al., 2017).

The effectiveness of maintaining energy metabolism is particularly related to the ability of fluoxetine to cross the mitochondrial membrane and the membranes of other cellular organelles (De Oliveira, 2016), implicating mitochondria as a possible site of drug action. This mechanism could be interesting in situations of damage, such as prenatal exposure to chronic stress, where the restoration of mitochondrial proteins involved in antioxidant defenses could offer a progressive improvement of the injured brain (Glombik et al., 2017). However, levels of protein expression do not necessarily reflect protein activity, so care is needed in the interpretation of these studies and making suggestions.

Mitochondrial functionality is comprised of several integrated functions, and as Peric et al., 2018 has mentioned, bioenergetics and the redox state are certainly important aspects to take into account when mitochondria are modulated by any stimuli. In that study, fluoxetine changed the proteomic patterns of the mitochondrial proteins in

the hippocampus of chronic social isolated (CSIS) rats, including upregulation of electron transport chain proteins, reversing the effects of CSIS (Peric et al., 2018). In addition to the modulation of oxidative phosphorylation, proteins involved in the metabolic process (i.e., glycolysis and the tricarboxylic acid cycle) were also targets of antidepressant effects, suggesting an important contribution to energy metabolism of brain areas affected by chronic stress.

Despite the differences in the experimental designs between studies, these studies all pointed out the known interaction of SSRI treatment with mitochondria and the modulation of its central structure function. Therefore, it is reasonable to mention that toxicity and safety are not easily manipulated and depend on a broad range of well-controlled variables such as time and route of drug treatment.

Taken together, this systematic review proposes future research in continuing to elucidate the effects of SSRI administration on the mitochondria and in identifying novel drug-organelle interactions in experimental models. This review contributes to a better understanding of the molecular effects of antidepressants in a wide context, not only in brain structures but also in other non-classic organs, with potential therapeutic motives that attempt to strengthen clinical data.

In summary, mitochondrial responses to serotonin reuptake inhibitor exposure in various brain regions was mostly related to metabolic and energetic consequences in the cell, supporting the relevance of this organelle in the pathophysiologic process of the central nervous system and its direct interaction with SSRI drugs. Therefore, understanding the molecular mechanisms associated with drug exposure can assist in

understanding the target mechanisms of pharmacological interventions in cellular bioenergetics modulation.

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5. Conflicts of interest

The authors report no conflict of interest.

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Table captions

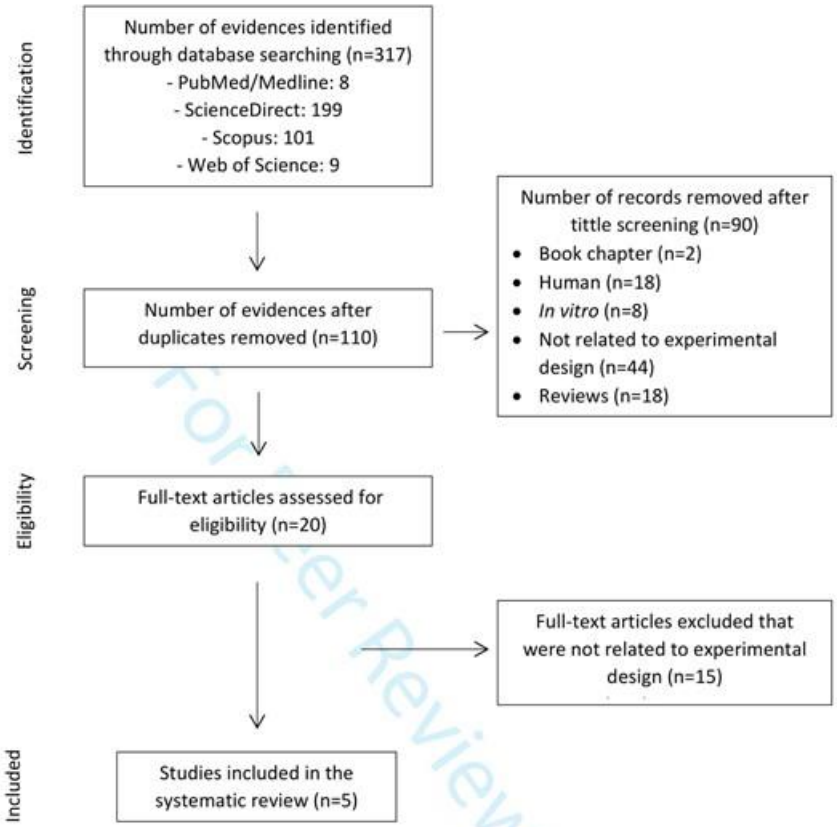
Table 1: Assessment of the risk of bias by SYRCLE'S tool of included studies. YES=bias described (indicating low risk of bias); NO=bias not described (indicating high risk of bias); UNCLEAR=indicating an unclear risk of bias.

Table 2: Main experimental and pharmacological characteristics of the included studies.

Table 3: Summarized information of mitochondrial analysis and main outcomes of the included studies. 3-NP = 3-Nitropropionic acid, CSIS = chronic social isolation, CUS = chronic unpredictable stress, L-NAME = N(ω)-nitro-L-arginine methyl ester, MTT = 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-H-tetrazolium bromide.

Figure caption

Fig 1. PRISMA flow diagram of systematic study selection



Item	Kumar et al., 2009	Kumar et al., 2010	Wen et al., 2010	Glombik et al., 2017	Peric et al., 2018
Sequence generation	NO	NO	NO	NO	NO
Baseline characteristics	YES	YES	YES	YES	YES
Allocation concealment	NO	NO	NO	NO	NO
Random housing	YES	YES	YES	YES	YES
Blinding of investigators	UNCLEAR	UNCLEAR	UNCLEAR	UNCLEAR	UNCLEAR
Random outcome assessment	UNCLEAR	UNCLEAR	UNCLEAR	UNCLEAR	UNCLEAR
Blinding of outcomes assessors	NO	NO	NO	NO	NO
Incomplete outcome data	YES	YES	NO	NO	NO
Selective outcome reporting	UNCLEAR	UNCLEAR	NO	NO	NO
Other bias	NO	NO	YES	NO	NO

Author, Year	Sex/Species	Age (Weight)	SSRI choice, dose	Route of administration	Total period of administration	Tissue analyzed
Kumar et al, 2009	Male *Wistar* rats	4-5 months-old (250-300g)	Sertraline (5 and 10mg/kg)	Oral route	14 days	Cortex, striatum and hippocampus
Kumar et al, 2010	Male *Wistar* rats	4-5 months-old (250-300g)	Sertraline (5, 10 and 20 mg/kg)	Oral route	14 days	Hippocampus
Wen et al, 2014	Male *Sprague-Dawley* rats	4 month-old (300-400g)	Fluoxetine (5mg/kg)	Intragastrically	18 days	Raphe nuclei
Glombik et, 2017	Male *Sprague-Dawley* rats	~2 month-old (200-250g)	Fluoxetine (10mg/kg)	Intraperitoneal	21 days	Hippocampus
Peric et al, 2018	Male *Wistar* rats	2.5 months-old	Fluoxetine (15 mg/kg)	Intraperitoneal	21 days	Hippocampus

Author, Year	Aim	Mitochondrial Analysis	Main Outcomes	Conclusion
Kumar et al, 2009	To explore the possible role of sertraline against 3-NP induced behavioral, biochemical and mitochondrial dysfunction.	Estimation of complex I, II and IV activities and indirect activity of mitochondrial complex III.	Sertraline reversed mitochondrial enzymes activity dysfunctions induced by 3-NP.	Sertraline modulated neurotoxicity in the brain and could be employed as a neuroprotective adjuvant to abrogate oxidative stress *in vivo*.
Kumar et al., 2010	To study the neuroprotective profile of antidepressants against 3-NP induced neurotoxicity with a possible contribution of NO mechanism.	Estimation of complex I, II and IV activities, and mitochondrial redox activity (by MTT method).	Sertraline (10 and 20mg/kg) restored mitochondrial enzymes and mitochondrial redox activities damaged by 3-NP and its efficacy (at 10mg/kg) was enhanced in the presence of the NO activator-L-NAME (10mg/kg).	Antidepressants, specially sertraline, improves mitochondrial activity partly mediated through NO pathways.
Wen et al., 2014	To compare exercise and fluoxetine effects on biochemical, behavioral and mitochondrial parameters of chronic stressed animals.	Mitochondrial respiration and ATP production	Fluoxetine decreased mitochondrial respiratory control rate and ATP production in depressed rats compared to their controls.	Fluoxetine treatment did not differ from exercise intervention on mitochondrial parameters, although this antidepressant negatively modulated mitochondrial function in depressed rats.

Glombik et, 2017	To compare the impact of chronic imipramine and fluoxetine treatment on the mitochondria-enriched subproteomic profile in the hippocampus of an animal model for depression	Mitochondria-enriched subproteomic profile	Fluoxetine treatment in prenatally stressed rats induced up-regulation in 12 proteins related mainly to structure, dynamic and metabolism while down-regulated 8 proteins involved most in electron transport chain.	The antidepressant treatment enhanced expression of proteins engaged in mitochondrial biogenesis and oxidative defense.
Peric et al, 2018	To investigate behavioral and hippocampal subproteomic changes in rats following CSIS in Fluoxetine-treated rats	Proteome patterns from non-synaptic mitochondria	Fluoxetine treatment in CSIS rats induced an up-regulation in several proteins including those involved in oxidative phosphorylation and metabolic pathways in mitochondria fraction.	Fluoxetine application to CSIS rats predominantly increases cells energy demands

5.2 Artigo 2 - Serotonin modulation in overfed rats improves hypothalamic mitochondrial respiration, reduces oxidative stress and induces mitochondrial biogenesis

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Manuscript Draft

Manuscript Number:

Title: Serotonin modulation in overfed rats improves hypothalamic mitochondrial respiration, reduces oxidative stress and induces mitochondrial biogenesis

Article Type: Research Paper

Section/Category: Molecular and cellular pharmacology

Keywords: electron transport chain complex proteins; overfeeding; oxidative phosphorylation; oxidative stress; RT-PCR; serotonin reuptake inhibitor.

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Abstract: Nutritional imbalance in early ages may disrupt central control of energy homeostasis in the hypothalamus and favors the onset of metabolic diseases. Brain serotonin (5-HT) system plays an important role in hypothalamic homeostatic control of energy balance but underlying mechanisms of 5-HT regulation of energy metabolism still are poorly described. Mitochondrial function is related to energy production, ROS generation and oxidative balance and can be modulated by stress adaptation mechanisms such as mitochondrial biogenesis. Due to the scarce evidence regarding the effects of serotonin reuptake inhibitors (SSRI) such as fluoxetine (FLX) in the mitochondrial function, we aimed to study the potential contribution of fluoxetine in targeting mitochondrial function and mitochondrial biogenesis in overfed rats. Following the neonatal overfeeding model by reduced litter size adjustment, male Wistar rats were divided into 4 groups according to pharmacological administration during PND 39 - PND 59: normofed + vehicle (NV), normofed + FLX (NF), overfed + vehicle (OV) and overfed + FLX (OF). We found that neonatal overfeeding impairs mitochondrial respiration and induces oxidative stress in the hypothalamus (e.g. increased ROS production, lipid peroxidation, protein oxidation and decreased REDOX state). FLX administration in overfed rats recovered mitochondrial oxygen consumption and increased Ucp2 expression. Besides, ROS production and oxidative stress biomarkers were reduced and mitochondrial biogenesis-related genes were up-regulated. Taken together our results suggest that FLX administration in neonatal overfed rats improves mitochondrial respiratory chain activity, oxidative balance and induces mitochondrial biogenesis transcriptional expression in the hypothalamus towards to a mitochondrial energy efficiency.

Serotonin modulation in overfed rats improves hypothalamic mitochondrial respiration, reduces oxidative stress and induces mitochondrial biogenesis

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Running title: Fluoxetine drives hypothalamic mitochondrial efficiency

Abstract

Nutritional imbalance in early ages may disrupt central control of energy homeostasis in the hypothalamus and favors the onset of metabolic diseases. Brain serotonin (5-HT) system plays an important role in hypothalamic homeostatic control of energy balance but underlying mechanisms of 5-HT regulation of energy metabolism still are poorly described. Mitochondrial function is related to energy production, ROS generation and oxidative balance and can be modulated by stress adaptation mechanisms such as mitochondrial biogenesis. Due to the scarce evidence regarding the effects of serotonin reuptake inhibitors (SSRI) such as fluoxetine (FLX) in the mitochondrial function, we aimed to study the potential contribution of fluoxetine in targeting mitochondrial function and biogenesis in overfed rats. Following the neonatal overfeeding model by reduced litter size adjustment, male *Wistar* rats were divided into 4 groups according to pharmacological administration during postnatal day (PND) 39 – PND 59: normofed + vehicle (NV), normofed + FLX (NF), overfed + vehicle (OV) and overfed + FLX (OF). We found that neonatal overfeeding impairs mitochondrial respiration and induces oxidative stress in the hypothalamus (e.g. increased ROS production, lipid peroxidation, protein oxidation and decreased REDOX state). FLX administration in overfed rats recovered mitochondrial oxygen consumption and increased *Ucp2* expression. Besides, ROS production and oxidative stress biomarkers were reduced and mitochondrial biogenesis-related genes were up-regulated. Taken together our results suggest that FLX administration in neonatal overfed rats improves mitochondrial respiratory chain activity, oxidative balance and induces mitochondrial biogenesis transcriptional expression in the hypothalamus towards to a mitochondrial energy efficiency.

Keywords: electron transport chain complex proteins, overfeeding, oxidative phosphorylation, oxidative stress, RT-PCR, serotonin reuptake inhibitor.

1. Introduction

During the life course, many of the health impairments that become noticeable has raised in early life. Thus, nutritional manipulation during neonatal ages has a powerful contribution in the onset and maintenance of long-term illness as obesity. In this critical period of the organism's development, sensitiveness to external cues is higher and adaptation is a key process to survival that may have detrimental effects to the tissue function throughout life (Gillman, 2010). In order to regulate body homeostasis and energy metabolism, hypothalamic function relies on different well-orchestrate pathways that dictate a positive or negative energy balance according to peripheral signals. This neurocircuitry relies on activation of anorexigenic or orexigenic neuron subpopulations (e.g POMC/CART and NPY/AgRP, respectively) to proper induce satiety and hungry effects (Heisler et al., 2006). Disruption of hypothalamic function by non-healthy nutritional habits as excess of calories may severely damage energy balance regulation and permanent affect body weight control (Bouret, 2009).

Along with the changes in energy balance neurocircuitry, mitochondria as the main organelle responsible for energy production remains as a potential target of obesity-related dysfunction. In a process known as oxidative phosphorylation, mitochondria couples oxygen consumption and ATP synthesis by complex V with generation of reactive oxygen species (ROS) as by products of oxygen consumption, mostly in the complex I and III (Mitchell et al., 1967). Mitochondrial dysfunction by overfeeding includes decreased oxygen consumption, increased ROS production and reduced oxidative balance that favors a pro-oxidant environment. Altogether this may reflect in an adaptation of mitochondrial function in order to restore energy levels in a process known as mitochondrial biogenesis (Valero, 2014). Coordination between nucleus and mitochondria by several transcriptional factors such as peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), nuclear respiratory factor (NRF) and mitochondrial transcriptional factor A (TFAM) characterize a positive signal to increase mitochondrial amount by stimulating mitochondrial respiratory chain proteins (Ventura-Clapier et al., 2008).

Due to the role of mitochondria in energy efficiency, pharmacological approaches to counteract mitochondrial dysfunction related to metabolic impairments can result in broad contribution in the experimental metabolism research. Among potential underlying

mechanisms, modulation of serotonin (5-HT) system is relatively new in mitochondrial studies although its contribution in energy balance regulation is not a novelty (Donovan et al., 2013; Marston et al., 2011). In this field, our research group have demonstrated that the serotonin reuptake inhibitor-SSRI (fluoxetine) is crucially involved in mitochondrial function by promoting bioenergetics efficiency, UCP activation, enzymatic and non-enzymatic antioxidant defense and lower ROS production in central and peripheral tissues of *Wistar* rats (Braz et al., 2016; Da Silva et al., 2015; Silva et al., 2018; Simoes-Alves et al., 2018). Such effects have guided us to investigate the potential contribution of fluoxetine on mitochondria in overfeeding conditions. Based on previous findings that a chronic FLX treatment in overfed rats resulted in a positive energy balance regulation with lower adiposity and food intake and higher expression of satiety-related neuropeptides in the hypothalamus (Da Silva et al., 2019) this study was conducted to continue the rationale that fluoxetine could act as a feasible metabolic activator and crucially drive mitochondrial improvements in the hypothalamus of overweight rats.

2. Materials and Methods

2.1 Animals

The present study was performed in accordance with the National Institutes of Health (NIH, Bethesda, USA) guidelines for animal care and has been approved by the local Ethics Committee for animal experimentation from the Health Sciences Center of the Federal University of Pernambuco (Process number: 0027/2017). Twenty *wistar* female rats were mated 2 female:1 male in a 12h-dark-ligt-cycle (dark 11 a.m.-11 p.m., 23 ± 1ºC) and received water and commercial chow (Labina – Presence®) *ad libitum*. At first postnatal day (PND) male pups with equal body weight (6-8g) were randomly assigned with 9 pups per dam. At PND 3, litter size was adjusted in either 9 pups (normofed group, n total = 27) or 3 pups (overfed group, n total = 27). By reducing litter size, animals have higher breastmilk availability and become overweight in lactational period and throughout their lifetime (Plagemann et al., 1992; Voits et al., 1996). With 21 days of age, weaned animals start to receive water and commercial chow *ad libitum* until 60 days of age.

120 2.2 *Pharmacological administration*

121 From the PND 39 through PND 59, previous groups were reassigned based on
122 subcutaneous pharmacological administration of vehicle solution (NaCl 0.9%, 10ml/kg
123 b.w.) or fluoxetine (FLX – 10 mg/kg b.w., in vehicle solution, 10 ml/kg b.w.) (Da Silva
124 et al., 2019). Four experimental groups were divided: (1) Normofed + vehicle solution
125 (NV, n=13) (2) Normofed + FLX (NF, n=14) (3) Overfed + vehicle (OV, n=13) and (4)
126 Overfed + FLX (OF, n=14).

127

128 *2.3 Mitochondrial preparation and oxygen consumption*

129 Following mitochondrial differential centrifugation as previously published
130 (Lagranha et al., 2010), hypothalamic mitochondria was then assayed by Bradford
131 protocol to protein quantification (Bradford, 1976). A total of 0.5mg/mL protein was used
132 for measure mitochondrial oxygen consumption in a 600-SL chamber with controlled
133 temperature (28 ºC) connected to a Clark-type oxygen electrode (Hansatech Instruments,
134 Pentney King's Lynn, UK). Oxygen consumption was performed with proper respiration
135 buffer (Da Silva et al., 2015) and substrates for complex I (glutamate 10mM and malate
136 2mM) and II (succinate 5mM + rotenone 4µM) recording basal state (mitochondria and
137 substrates), ADP-stimulated state (ADP 0.5mM/L), resting state (oligomycin 1.2 mM/L)
138 and uncoupling state (CCCP 1mM/L). As a functional indicator of mitochondrial activity,
139 respiratory control rate (RCR) was calculated by the ratio between basal and
140 phosphorylation state. Data were expressed as nmol of oxygen/min/mg of protein.

141

142 *2.4 Mitochondrial reactive species (RS) production*

143 To measure mitochondrial total reactive species production, we used 1 µM of
144 fluorescent probe DCFDA (5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein
145 diacetate, acetyl ester) incubated with 0.5mg/mL of mitochondria and respiration buffer
146 (28 ºC, pH 7.2) (Da Silva et al., 2015). Fluorescence intensity was recorded for 5 min
147 using a spectrofluorimeter (OMEGA, USA) at 485 nm excitation and 530 nm emission.
148 RS production was estimated by slop from the area under the curve.

149

2.5 Tissue preparation and protein quantification

Another set of hypothalamic tissue was prepared for oxidative stress analysis. Briefly, hypothalamus was homogenized using TRIS buffer 50mM, pH 7.4, EDTA 1mM, sodium orthovanadate 1mM and PMSF 100 µg/mL using a Ultra-Turraz homogenizer (modelT10 BS32; IKA,Germany). Samples were then centrifuged at 4ºC, 461g for 10 min and the supernatant submitted to protein quantification by Bradford protocol (Bradford, 1976). The following analyses of oxidative balance were conducted with 0.3mg/mL of total protein.

2.6 Lipid peroxidation assay

To determine lipid peroxidation in the hypothalamus, TBARS method was performed according to Buege and Aust protocol (Buege et al., 1978). Samples were mixed with 30% trichloroacetic acid (TCA) in equal volumes and Tris-HCl buffer (10mM, pH 7.4) and centrifuged at 2500g for 10min. Next, supernatant and 0.73% thiobarbituric acid (w/v) were boiled at 100 ºC and the pink pigment was measured at 535nm in a spectrophotometer (Even, Model IL-512, UV/VIS). Data were expressed as mmol/mg protein.

2.7 Protein oxidation assay

To determine protein oxidation in the hypothalamus, Carbonyl content was assayed by Levine's protocol (Levine et al., 1990). 30% TCA and samples (w/v) were mixed and centrifuged at 4000rpm for 15min. The pellet was resuspended in 10 mM 2.4 dinitrophenylhydrazine and incubated at RT in the dark for 1h. Three times wash in ethyl/acetate solution were alternated with centrifugation at 4000rpm for 5min and the final pellet was incubated at 37 ºC for 30 min after mixing with 6M guanidine hydrochloride. Solution was submitted to spectrophotometer (Even, Model IL-512, UV-VIS) reading at 370nm and data were expressed as nmol/mg protein.

2.8 REDOX status

Reduced glutathione (GSH) and oxidized glutathione (GSSG) content were evaluated according to Hissin and Hilf protocol (Hissin et al., 1976) by incubation with 1 mg/mL O-Phthalaldehyde (OPT) at RT. For GSSG content, however, samples were prior incubated with 0.04M N-ethylmaleimide for 30 min in RT. Fluorescence intensity was recorded for 5 min using a spectrofluorimeter (OMEGA, USA) at 350 nm excitation and 420 nm emission and compared to a standard GSH and GSSG curves at known concentration. Data were expressed as µmol/mg of protein. REDOX state was presented as the ratio between GSH and GSSG content.

2.9 Oxy-score

As an estimative of a global oxidative status, the biomarkers of oxidative balance described above were used to calculate the oxy-score. Using the following equation, Oxy-score=ANTIOX-OXY, where ANTIOX measurements correspond to the sum of standardized antioxidant results (GSH) and OXY is equivalent to the sum of standardized oxidative results described (MDA, Carbonyls and GSSG content) (Braz et al., 2017). A positive oxy-score indicates prevalence of antioxidant capacity while negative scores suggest an oxidative damage. Data were expressed as arbitrary units.

2.10 RT-PCR for mitochondrial biogenesis-related genes

Total RNA was extracted from iBAT, rtWAT and eWAT using TRIZOL reagent (Invitrogen, USA) and guanidine isothiocyanate/phenol method (Chomczynski et al., 1987) for mRNA expression of the following genes: Beta -2 microglobulin (*β2M*), Uncoupling protein 2 (*Ucp2*), AMP-activated protein kinase (*Ampk*), Peroxisome activated receptor gamma coactivator 1 alpha (*Pgc1α*), *Nuclear respiratory factor 2* (*Nrf2*), Mitochondrial transcriptional factor A (*Tfam*), NADH:Ubiquinone Oxidoreductase Subunit B8 (*Ndufb8*), Succinate Dehydrogenase Complex Iron Sulfur Subunit B (*Sdhb*) and ATP synthase alpha-subunit (*Atp5a*). RNA concentration was determined with Nanodrop (Thermo Scientific, USA) and RNA purity was assessed by 260/280 ratio (>1.8). RT-PCR was performed using Rotor-Gene Q (Qiagen, USA) as previously described (Da Silva et al., 2019) running three samples by group in duplicate.

209 Cycle threshold (CT) of each targeted gene (Table 1) was compared with CT of internal
210 control. Relative mRNA expression is shown as $2^{-\Delta\Delta Ct}$ (Livak et al., 2001).

211

212 *2.11 Statistics*

213 Data were tested to normality through Kolmogorov-Smirnov analysis and running
214 with two-way ANOVA test followed by Tukey's post hoc test to assess differences among
215 groups. Results are expressed as percentage from control group with $p<0.05$ considered
216 statistically significant. All analysis was performed using GraphPad Prism 6.0® software
217 (GraphPad Prism Software, Inc., La Jolla, CA, USA).

218

219 **3. Results**

220 *3.1 Mitochondrial oxygen consumption and Ucp 2 expression*

221 Related to the hypothalamic mitochondrial respiration, when mitochondria were
222 stimulated with complex I substrates (Fig 1A), the oxygen consumption of overfed group
223 showed decreased ADP-stimulated phosphorylation capacity (33%, $p<0.0001$, n=5)
224 compared to NV group. Fluoxetine treatment different modulated mitochondrial
225 respiration, though. While NF group showed even lower phosphorylation capacity when
226 compared to control group (68%, $p<0.0001$, n=5), OF group showed a decrease in basal
227 state (59%, p=0.0436, n=5) and resting state (52%, p=0.0127, n=5) and an increased
228 uncoupling capacity (30%, p=0.0354, n=5) compared to OV group. RCR with complex I
229 substrates (Fig 1A-insert) was significantly decreased in OV group compared to NV
230 group (62%, p=0.0046, n=5) and FLX treatment significantly decreased RCR in NF group
231 compared to NV group (81%, p=0.0005, n=5) and markedly increased this measurement
232 in OF group compared to OV group (140%, p=0.0135, n=5). When analyzing oxygen
233 consumption with complex II substrates (Fig 1B), we demonstrated that there was an
234 impairment in hypothalamic mitochondria of the OV group compared to NV group,
235 independent of the substrate used, indicated here by increased basal state (58%, p=0.049,
236 n=5), decreased ADP-phosphorylated state (33%, p=0.0018, n=5), increased resting state
237 (68%, p=0.044, n=5), and decreased uncoupling state (53%, $p<0.0001$, n=5). NF group
238 showed decreased uncoupling capacity (27%, $p<0.0001$, n=5). In contrast to OV group

though, OF group improved overall oxygen consumption, presenting lower basal (20%, p=0.041, n=5) and resting states (22%, p=0.049, n=5) and higher ADP-stimulated (63%, p<0.0001, n=5) and uncoupling sates (52%, p<0.0001, n=5). RCR with complex II substrates (Fig 1B - insert) was 60% lower in the OV group than NV group (p=0.0034, n=5) and 122% higher in OF group compared to OV group (p=0.0056, n=5). To better understand the changes in mitochondrial oxidative phosphorylation we next investigate whether the proton leak independent of ATP-synthase would be modulated by our experimental model. Thus, mitochondrial uncoupling protein 2 (UCP 2) was assayed for gene expression (Fig 1C) and we showed that OF group increased *Ucp2* expression compared to OV group (146%; p=0.0059, n=4).

3.2 Mitochondrial ROS production and Oxidative stress biomarkers

Related to mitochondrial reactive species (RS) production (Fig 2A) our results showed that in agreement with the mitochondrial respiration impairments, OV group had a 93% increase in RS production compared to NV group (p=0.0098, n=4). NF group also had an increase compared to NV group (109%, p=0.0040, n=4) while OF group had 39% less RS production compared to OV group (p=0.0292, n=4). To determine the extent of oxidative stress in the hypothalamus several parameters were measured. Lipid peroxidation, measured by MDA concentration (Fig 2B), was increased in OV group (111%, p=0.0002, n=5) compared to NV group. Similarly, NF group increased MDA concentration compared to NV group (73%, p=0.0095, n=4). On the other hand OF group showed less lipid peroxidation than OV group (26%, p=0.0299, n=5). Carbonyls content (Fig 2C) related to protein oxidation, was increased in OV group compared to NV group (68%, p=0.0390, n=5) and decreased in OF group compared to OV group (49%, p=0.0062, n=5). In the non-enzymatic defense, the main intracellular antioxidant thiol, GSH (Fig 2D), was reduced in OV group by 42% (p<0.0012, n=5) and in NF group by 27% (p=0.0416, n=5) when compared to NV group. OF group increased GSH levels in 64% compared to OV group (p=0.0057, n=5). GSSG concentration (Fig 2E) reached a 3-fold-increase in OV group compared to NV group (p<0.0001, n=4) while decreased about 75% in OF group compared to OV group (p<0.0001, n=6). REDOX status (Fig 2F) was significantly impaired in OV group (90%, p<0.0001, n=4) and in NF group (30%, p=0.0489, n=4) compared to NV group. OF group though had a remarkable 8-fold-

increase compared to OV group ($p<0.0001$, n=4). Oxy-score results (Fig 2G), performed to assess a global antioxidant/pro-oxidant capacity of tissue, followed the pattern observed in REDOX status: reduced scores in OV group compared to NV group (75%, $p<0.0001$, n=7) and in NF group compared to NV group (67%, $p<0.0001$, n=7), with a greatly restoration of this measurement in OF group compared to OV group (239%, $p<0.0001$, n=7).

3.3 Mitochondrial biogenesis-related genes

Given that oxygen consumption and oxidative stress can regulate mitochondrial biogenesis, we next investigated changes in gene expression related to this matter. *Ampk* expression increased about 135% in NF group compared to NV group ($p=0.0142$, n=4). OF group also showed higher *Ampk* content compared to OV group (about 170%, $p<0.0001$, n=4). Regulation of *Pgc1α* was only observed in OF group, with a 47% increase compared to OV group ($p=0.0411$, n=4). *Nrf2* expression was higher in OV group (41% compared to NV group, $p=0.0108$, n=5) and in OF group (21% compared to OV group, $p=0.050$, n=5). *Tfam* expression was modulated only in OF group, with a 104% increase compared to OV group ($p=0.0331$, n=4). The complex I subunit *Ndufb8* expression had an increase of 117% in OV group compared to NV group ($p=0.0258$, n=4). (Fig 3). *Sdhb* expression, complex II subunit, increased in OF group compared to OV group (117%, $p=0.0035$, n=4). Ultimately, *Atp5a* expression, complex V subunit, increased in NF group compared to NV group (256%, $p=0.0232$, n=4) and in OF group when compared to OV group (83%, $p=0.0222$, n=4).

4. Discussion

Hypothalamic serotonergic neurons belong to the main central system of homeostatic control of energy balance that receive peripheral nutrient-related signals in order to adjust the integrative response accordingly (Heisler et al., 2006). Mitochondria as suitable organelles that dictates overall metabolism display important activities specially in the brain regarding not only bioenergetics as synaptic transmission, calcium homeostasis, and neuronal excitability, all of them regulated by stress adaptation (Fanibunda et al., 2019). Herein we demonstrated that neonatal overfeeding impairs

mitochondrial respiratory activity and oxidative balance in the hypothalamus of juvenile rats and that chronic fluoxetine administration in overfed rats rescue those parameters besides induces transcriptional expression of genes related to energy sensing and mitochondrial biogenesis.

Mitochondrial dysfunction observed in overfed rats suggests that excess of calories may be detrimental for phosphorylation capacity which could negatively affect overall metabolism. Disruption of hypothalamic energy balance related-neuropeptides was recently demonstrated by our previous study (Da Silva et al., 2019) and the mitochondria data presented here corroborates with that, disrupts the mitochondrial essential role in energy metabolism contributing to the onset and/or maintenance of the overweight/obesity. Carraro et al., 2018 evaluating hypothalamic mitochondria failed to show respiration impairments in 1.5-month-old Swiss mice submitted to a high-fat diet. This apparently discrepancy may relies on diet short-term period (7 days) (Carraro et al., 2018). The adaptive response of mitochondria to obesity experimental models and its dysfunctional characteristics have been demonstrated before in rodents (Thaler et al., 2012; Bournat et al., 2010; Petrov et al., 2015), but concerning neonatal overfeeding conditions and its effects on brain regions we still have scarce reports.

By the contrast, modulation of the mitochondrial respiratory chain activity by fluoxetine in overfed rats suggests an improvement in the coupling between oxidative phosphorylation and energy production and hence increased respiratory control rate independent of the substrate used (i.e. glutamate/malate-complex I or succinate-complex II). These data were reinforced by increased *Ucp2* expression in the OF group and suggest that fluoxetine administration may have positive effects on mitochondrial membrane potential towards with less production of reactive oxygen species. Previous studies from our research group have pointed out that FLX administration in lactation period improves mitochondrial respiration and reduces ROS generation in the hypothalamus of 60-day-old male rats (Da Silva et al., 2015). Applying similar pharmacological model (PND1-PND21, 10 mg/kg b.w.), Silva et al., 2018 demonstrated that FLX induced increased *Ucp2* expression and similar improvements in mitochondrial bioenergetics and ROS production in the brainstem of male rats with 60 days of age (Silva et al., 2018). Since literature have not reported fluoxetine effects on mitochondrial bioenergetics in the hypothalamus of overweight/obese rats, our data contributes to clarify the therapeutic effects of the

334 modulation of brain serotonin system in experimental overweight/obesity models clearly
335 seen in overfed but not in normofed group.

336 Mitochondria is considered the main ROS generator in the brain and this is well
337 correlated with energy balance control specially in the homeostatic signaling coordinated
338 by the hypothalamus (Williams, 2012). Obesity-associated mitochondrial dysfunction
339 leads to excessive reactive oxygen species generation and this was observed here in OV
340 animals that along with mitochondrial impairments displayed higher lipid peroxidation
341 and protein oxidation and lower REDOX status. Offering a high-fat diet (HFD) to young
342 male rats Cavaliere et al., 2018 reported increased hypothalamic ROS production, lipid
343 peroxidation and reduced REDOX state (Cavaliere et al., 2018). This study provided a
344 long-term HFD evaluated in different period and after 3-weeks of diet, comparable period
345 with our model, the similar oxidative stress condition has contributed to oxidative balance
346 disruption. This has been proposed as a key mechanism correlated to the damage of
347 cellular biomolecules including DNA. In the brain, the most vulnerable tissue to the
348 oxidative stress harmful effects (Halliwell, 2006), such oxidative damage may contributes
349 to inducement of energy metabolism deficiency and neurodegenerative diseases
350 (Pugazhenthi et al., 2017).

351 The effects of FLX administration in overfed rats reestablished all of the oxidative
352 balance disruptions attributed to overfeeding. This improvement was expected, once that
353 along with mitochondrial respiratory improvements our group has been published similar
354 results in neonatal FLX-treated rats in brain tissues (Braz et al., 2016; Da Silva et al.,
355 2015; Silva et al., 2018; Da Silva et al., 2014). Although not evaluated here, the
356 antioxidant potential of FLX was described before and close related to improved
357 antioxidant defense such as positive modulator of the antioxidant enzymes activities such
358 as, superoxide dismutase (SOD), catalase (CAT) and glutathione-dependent system
359 (glutathione-S-transferase-GST and glutathione peroxidase-GPx) (Da Silva et al., 2015;
360 Rebai et al., 2017). Their associated functions dictate the extent of the ROS damage that
361 also rely on non-enzymatic defense, i.e. reduced glutathione-GSH. This main intracellular
362 thiol is involved in activation of antioxidant enzymes activities, signal transduction, gene
363 expression, cell proliferation and apoptosis (Sies, 1999) and the major non-enzymatic
364 protector of central nervous system ROS-dependent impairments (Moretti et al., 2012).
365 In this sense we believe that a REDOX signaling (mediated not only by GSH/GSSH ratio
366 as well as by ROS signaling and *Ucp 2* expression) is implicated in the improvements of

oxidative balance and mitochondrial function induced by chronic FLX administration in overfed rats. Since the antidepressants are not aimed for obesity therapy, studies have been focused on the classic effects of SSRI and its antioxidant potential. In this sense FLX exposure *per si* (3 weeks, 15mg/kg b.w.) in 75-day-old male *Wistar* rats increased hippocampal GSH-dependent enzymes activity (GPx and glutathione reductase-GLR) in a study made by Peric et al., 2017 (Peric et al., 2017).

Because both mitochondrial oxidative phosphorylation and ROS generation are known modulator factors of mitochondrial biogenesis, we performed several RT-PCR assays to investigate whether nutritional and pharmacological interventions could induce transcriptional alteration in mitochondrial biogenesis-related agents. We found that overfeeding induced increased expression of *Nrf2* and the mitochondrial complex I subunit, *Ndufb8*. *Nrf2* is known to be a key transcriptional factor that induces antioxidant enzymes expression in the nucleus (Lin et al., 2006). We believe that this could be an adaptative protection to counteract the effects of overfeeding in the mitochondrial respiration impairments and ROS production with increased oxidative stress. FLX administration positive induced molecular pathways towards to mitochondrial biogenesis. As an energy sensor and regulator of energy homeostasis, hypothalamic *Ampk* is involved in many central and peripheral regulations that contributes to overall metabolic efficiency, including reduced food intake and increased thermogenesis of brown adipose tissue (Lopez, 2017), both factors included in our previous studies (Da Silva et al., 2019; Braz et al., 2020). Ampk signaling pathway was positive modulated by FLX in overfed rats, which further may have activated *Pgc1*α and that by stimulating *Nrf2* and *Tfam* expression, favored the nuclear signaling driven to mitochondrial targeting adaptations such as increased respiratory capacity and antioxidant defense. Activation of mitochondrial biogenesis supports the idea that molecular adjustments modulated by FLX are likely achieved to stimulation of the unsatisfactory mitochondrial energy metabolism found in the hypothalamus of overfed rats. Importantly, such molecular regulation mediated by FLX in the hypothalamus of overfed rats was made towards mitochondrial respiratory chain efficiency, mainly complex II, with substantial increment of both chemical (*ATP-synthetase* expression and related activity) and thermic (*Ucp2* expression) energy production.

Little is known about underlying mechanisms of SSRI effects on mitochondrial biogenesis but some studies have described increased mitochondrial biogenesis mediated

by serotonin receptors. In Parkinson's like disease experimental model, Scholpa et al., 2018 reported that 5-HT1F receptor agonist treatment (2mg/kg b.w. 14 days) in C57BL/6 mice increased mRNA expression of *Pgc1α* and *Ndufs1* (complex I subunit) in the frontal cortex and hippocampus and cytochrome c oxidase (COX) in the hippocampus, along with PGC1α and TFAM protein expression in different brain areas (Scholpa et al., 2018). This may provide a brain mitochondrial adaptation to the drug-induced abnormalities in this Parkinson's-like disease model and may regulate mitochondrial homeostasis towards brain metabolism improvement. Peric et al., 2018, studied 2.5-month-old *Wistar* rats that underwent a chronic stress events and were treated later with fluoxetine (15mg/kg b.w., 21 days). They demonstrated hippocampal up-regulation of proteins related to mitochondrial biogenesis, such as mitochondrial complex I, III and ATP synthase subunits (Peric et al., 2018). In this case, depression-like behaviors could also be favored by improving mitochondrial function and energy metabolism mediated by antidepressants.

Taken together our data suggests that neonatal overfeeding induces mitochondrial and oxidative impairments that were counteracted by a chronic serotonin modulation between PND 39 - PND 59that not only improved mitochondrial respiration and oxidative balance as well as induced mitochondrial biogenesis transcriptional factors in the hypothalamus of those treated-animals.

4. Conclusion

Fluoxetine treatment in overfed rats improves mitochondrial function and oxidative balance with inducement of important signaling pathway for mitochondrial biogenesis and metabolism suggesting an improvement in hypothalamic energy homeostasis at functional and molecular level. These contributes to progress in uncovering 5-HT pharmacological manipulation and its contribution to metabolism efficiency in experimental overfeeding model.

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Declaration of interest

None

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Table 1: Primers specifications

Gene	Sequence	Annealing temperature
Beta -2 microglobulin (ß2M)	Sense: TGA CCG TGA TCT TTC TGG TG Antisense: ACT TGA ATT TGG GGA GTT TTC TG	48 °C
Uncoupling protein 2 (Ucp2)	Sense: TAC TCT CCT GAA AGC CAA CC Antisense: GCT GCT CAT AGG TGA CAA AC	49 °C
AMP-activated protein kinase 1 (Ampk-1)	Sense: ACC ATT TAA CTC GGC CTC AC Antisense: TTG CTC TAC ACA CTT CTG CC	48 °C
Peroxisome activated receptor gamma coactivator 1 alpha (Pgc1α)	Sense: AAC AGC AAA AGC CAC AAA GA Antisense: AAG TTG TTG GTT TGG CTT GA	48 °C
Nuclear respiratory factor 2 (Nrf2)	Sense: CTT CCA TTT ACG GAG ACC CA Antisense: CCA GAA GAA TGT GTT GGC TG	49 °C
Mitochondrial transcription factor A (Tfam)	Sense: TCT CAT GAT GAA AAG CAG GCA Antisense: GAG ATC ACT TCG CCC AAC TT	48 °C
NADH:Ubiquinone Oxidoreductase Subunit B8 (Ndufb8)	Sense: TAG GAC CCC AGA AGA ACG GG Antisense: CGG TTA GGG AGC ATC GGG TA	51 °C
Succinate Dehydrogenase Complex Iron Sulfur Subunit B (Sdhb)	Sense: TTT ACC GAT GGG ACC CGG AC Antisense: CGT GTT GCC TCC GTT GAT GT	52 °C
ATP synthase alpha-subunit (Atp5a)	Sense: TCC CTG AAC TTG GAA CCC GA Antisense: GGC ATT TCC CAG GGC ATC AA	51 °C

566

567 **Figure Captions**

568 **Fig. 1: Mitochondrial oxygen consumption in the hypothalamus of male *Wistar* rats**
569 **from NV, NF, OV and OF groups at 60 days of age.** (A) mitochondrial respiration with
570 complex I substrates, (B) mitochondrial respiration with complex II substrates, (C) *Ucp2*
571 expression. Data presented as mean±SEM. All data were compared by two-way ANOVA
572 with Tukey's test for multiple comparisons. *p≤0.05, **p≤0.01, *** p≤0.001. White:
573 normofed + vehicle group; Gray: normofed + fluoxetine group; Black: overfed + vehicle
574 group and Blue: overfed + fluoxetine group.

575

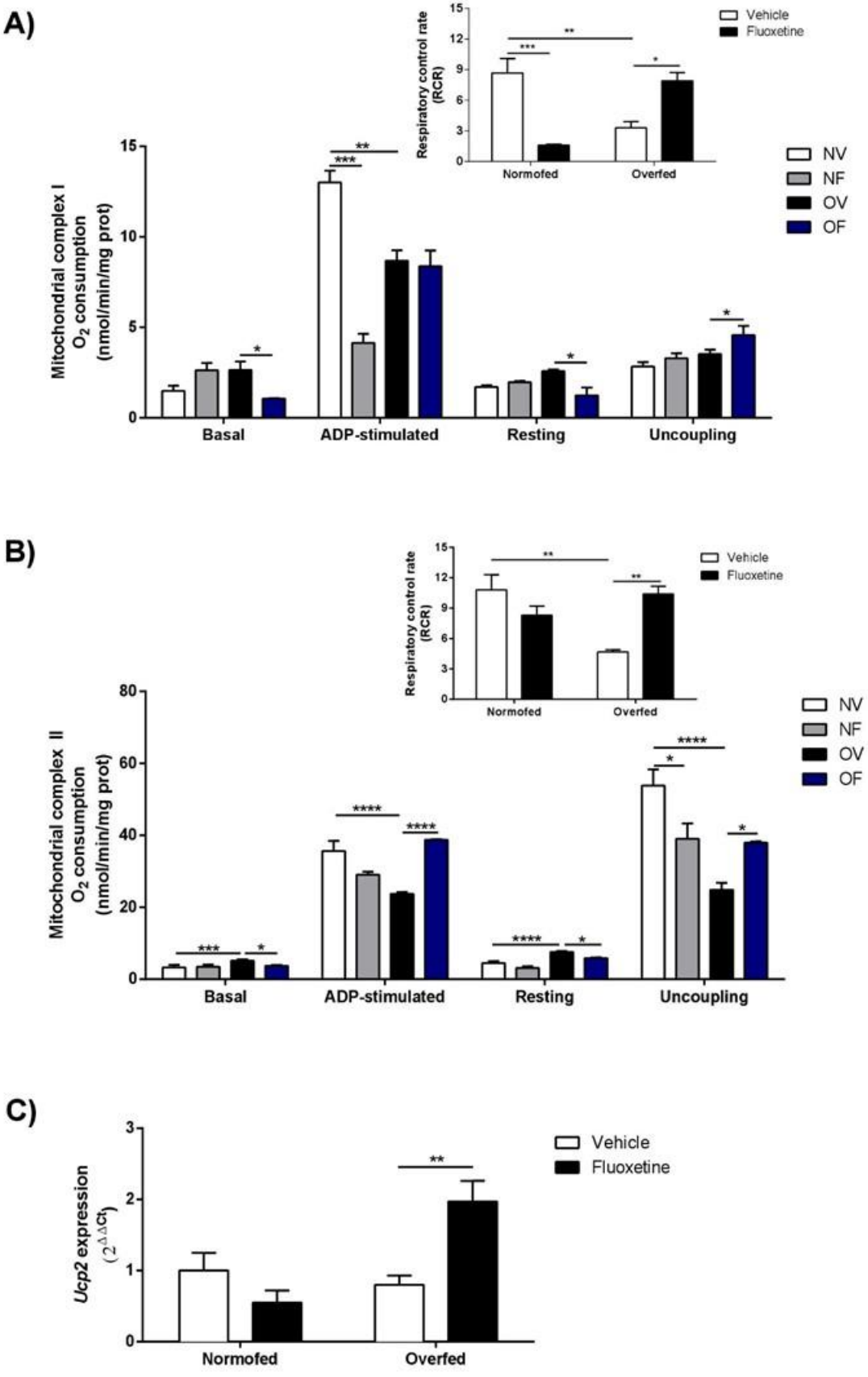
576 **Fig. 2: Oxidative stress biomarkers in the hypothalamus of male *Wistar* rats from**
577 **NV, NF, OV and OF groups at 60 days of age.** (A) Mitochondrial RS production, (B)

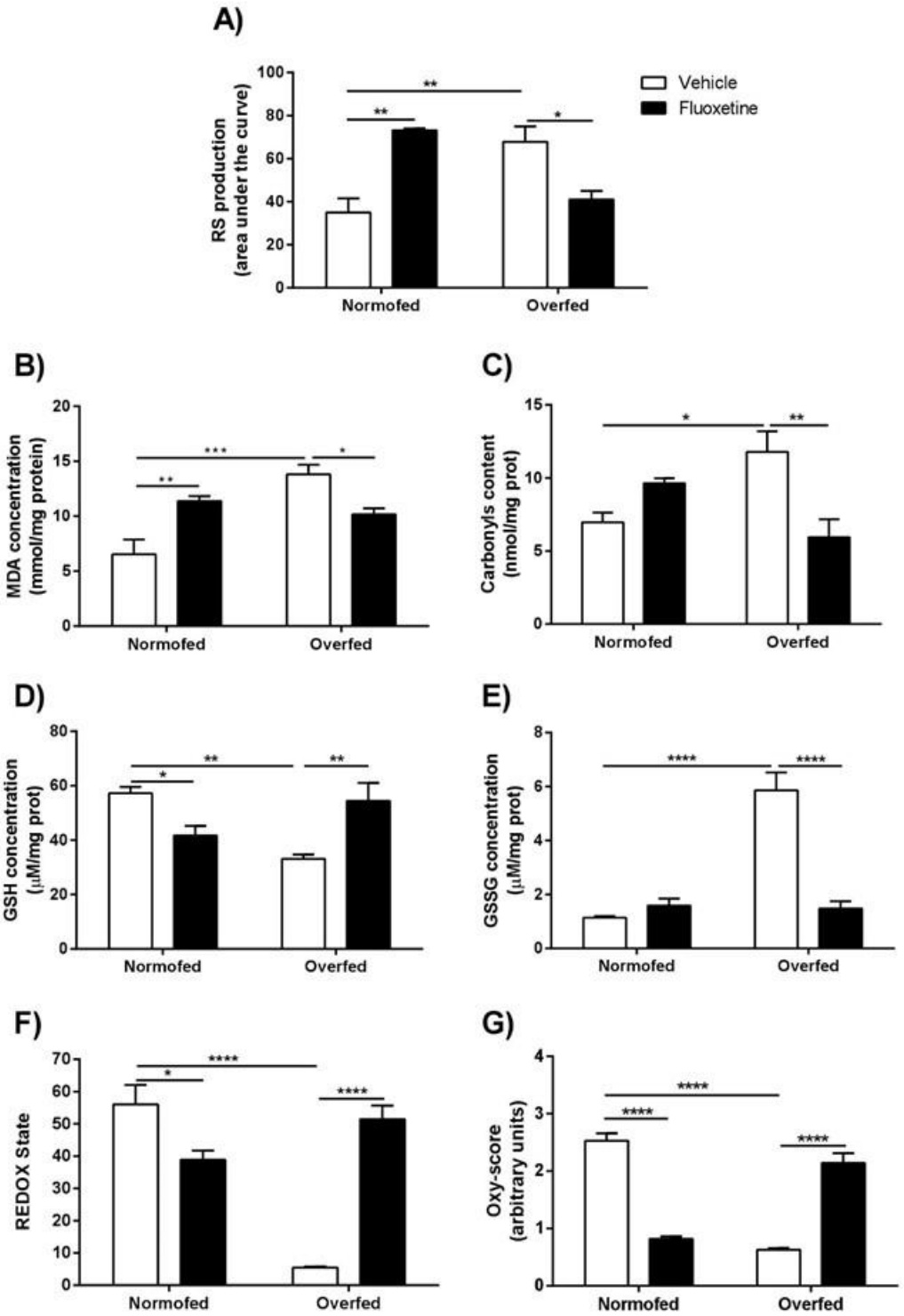
578 MDA concentration, (C) Carbonyl content, (D) GSH content, (E) GSSG content, (F)
579 REDOX state (Ratio between GSH and GSSH) and (G) Oxy-score (Ratio between
580 antioxidant and pro-oxidant measurements of oxidative stress). Data presented as
581 mean±SEM. Groups were compared by two-way ANOVA with Tukey's test for multiple
582 comparisons. *p≤0.05, **p≤0.01, *** p≤0.001, **** p≤0.0001.

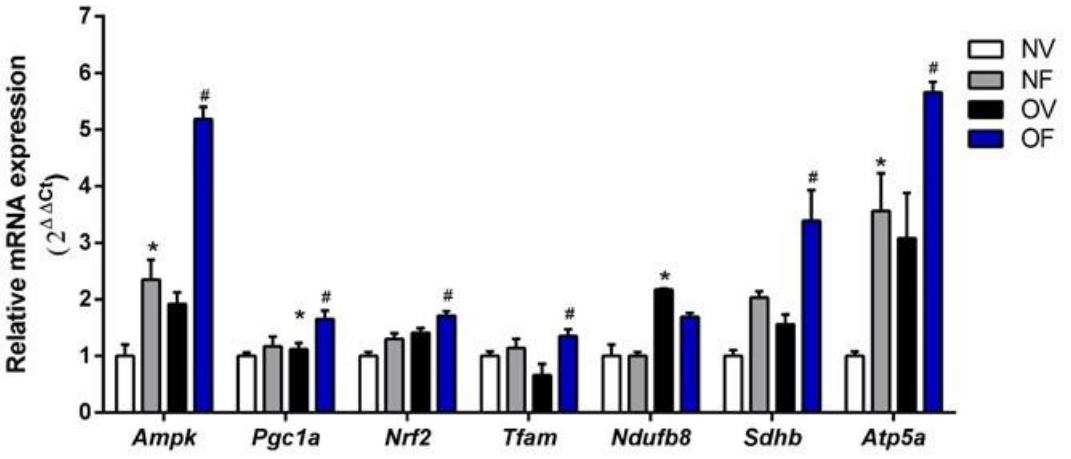
583

584 **Fig. 3: Gene expression of mitochondrial biogenesis agents in the hypothalamus of**
585 **male *Wistar* rats from NV, NF, OV and OF groups at 60 days of age.** Data presented
586 as mean±SEM, Groups were compared by two-way ANOVA with Tukey's test for
587 multiple comparisons. *p≤0.05 vs NV group, #p≤0.05 vs OV group. White: normofed +
588 vehicle group; Gray: normofed + fluoxetine group; Black: overfed + vehicle group and
589 Blue: overfed + fluoxetine group.

590


A)
Vehicle
Fluoxetine
Respiratory control rate (RCR)
Normofed
Overfed
Mitochondrial complex I O2 consumption (nmol/min/mg prot)
NV
NF
OV
OF
Basal
ADP-stimulated
Resting
Uncoupling
B)
Mitochondrial complex II O2 consumption (nmol/min/mg prot)
C)
Ucp2 expression (2^-ΔΔCt)


A)
RS production (area under the curve)
Vehicle
Fluoxetine
Normofed
Overfed
B)
MDA concentration (mmol/mg protein)
C)
Carbonyls content (nmol/mg prot)
D)
GSH concentration (µM/mg prot)
E)
GSSG concentration (µM/mg prot)
F)
REDOX State
G)
Oxy-score (arbitrary units)


Relative mRNA expression
($2^{\Delta\Delta Ct}$)
0
1
2
3
4
5
6
7
NV
NF
OV
OF
*
#
Ampk
Pgc1a
Nrf2
Tfam
Ndufb8
Sdhb
Atp5a

5.3 Artigo 3 – Chronic serotonin reuptake inhibition uncouples brown fat mitochondria and induces beiging/browning process of white fat in overfed rats

Journal Pre-proof

Chronic serotonin reuptake inhibition uncouples brown fat mitochondria and induces beiging/browning process of white fat in overfed rats

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Chronic serotonin reuptake inhibition uncouples brown fat mitochondria and induces beiging/browning process of white fat in overfed rats

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Running title: Fluoxetine drives mitochondrial efficiency and browning

Abstract

Aim: To investigate whether a chronic 5-HT reuptake inhibitor (i.e. Fluoxetine-FLX) exposure in young adult rats overfed during suckling period would modulate interscapular brown adipose tissue (iBAT) mitochondria and browning agents in white adipose tissue (WAT).

Methods: Male *Wistar* rats were assigned into either a normofed group (n=9 per group) or an overfed group (n=3 per group) induced by litter size reduction at postnatal day 3 (PND3). Pharmacological manipulation was carried out between PND39 and PND59 and groups were assigned accordingly: Normofed + vehicle solution – NaCl 0.9% (NV group), Normofed + FLX solution – 10mg/kg b.w. (NF group), Overfed + Vehicle (OV group) and Overfed + FLX (OF group). We evaluated mitochondrial oxygen consumption and reactive species (RS) production, oxidative stress analyzes (MDA concentration, carbonyl content, REDOX state [GSH/GSSG], global oxy score) in the iBAT, gene (*leptin*, *Ucp1*, *Sirt1*, *Pgc1α* and *Prdm16*) and protein (UCP1) expression in the iBAT and epididymal WAT (eWAT).

Key findings: OV group increased body weight gain, Lee index and oxidative stress in the iBAT. Both FLX-treated groups showed less weight gain compared to their controls. OF group showed different leptin expression in the WAT and iBAT; increased functional UCP1 content and mitochondrial activity with less oxidative stress in the iBAT and upregulation of browning genes in eWAT (*Pgc1α*, *Prdm16* and *Ucp1*).

Conclusion: Altogether our findings indicated that FLX treatment in young adult overfed animals improved the iBAT mitochondrial function, reduced oxidative stress and induced transcriptional activation of browning agents in white adipose tissue.

Keywords: mitochondria, brown adipose tissue, SSRI, beige fat, browning, oxidative stress.

1. Introduction

The poor quality of early nutrition has reflected in the children nutritional state once overweight/obesity has reached 38 million children aged under five-years-old [1]. According to the World Health Organization reports, childhood obesity became an epidemy far from being a controlled public health issue with the tendency to increase the number of overweight/obese individuals almost twice as higher the currently estimative [1].

Overweight/obesity is detrimental for several metabolic tissues like liver, skeletal muscle, heart and brain [2-5]. The dysfunctional characteristic though can rely, but not only, on the extent of the nutritional insult and the age and period in which that insult occurred. Interestingly, the brown adipose tissue (BAT) has been targeted by obesity researches due to the critical thermogenic action involved in body homeostasis, mainly upon cold stimuli and endocrine factors that drive sympathetic outflow onto BAT [6]. Attributed to the BAT function thought, newborns have comparable large amounts of BAT than adult, but previous reports demonstrated that even in the adulthood, BAT was found in detectable amounts supported by PET-CT data, with functional mitochondrial uncoupling protein 1 (UCP1) [7]. Such information has attracted attention regarding modulation of BAT function and the potential metabolic contribution in anti-obesity therapeutics.

Because brown fat cells function is intrinsic related to their mitochondrial activity, this organelle is essential to cellular and overall metabolism. In this sense, UCP1, a BAT specific inner mitochondrial membrane protein allows the dissipation of the proton conductance as heat by the oxidation of fatty acids in the mitochondrial respiratory chain [8]. Moreover, regulation of oxidative stress process in mitochondria of brown fat tissue direct contributes to the oxidative balance, reactive oxygen species (ROS) production and maximal mitochondrial respiration capacity may contributing to the thermogenic activity [9]. Evidences suggest that obesity and/or its metabolic dysfunctions may have modulate BAT activity, mitochondrial function, oxidative balance and inflammatory regulation [9-11], however the extent of early nutritional imbalance on mitochondrial oxidative function and energy balance regulators in the adult BAT remains a scarce research field.

Strategies to modulate adipose function towards an efficient energy metabolism in the course of overweight/obesity has an outstanding role in the future of disease approaches. Serotonin (5-HT) system, among several mechanisms, represents an interesting subject to be explored given the close relationship with energy balance control. Well known to positively modulate feeding behavior, 5-HTergic system display a pivotal role in obesity control, where by peripheral or central modulation, adiposity and other obesity-related

parameters are reduced [12, 13]. Serotonin reuptake inhibitors (SSRI), as fluoxetine (FLX), are widely used antidepressants that reduce body weight gain through central modulatory mechanisms, although such effect still is controversial when considering long-term effects. Listed previously as a candidate in the anti-obesity therapeutic studies [14, 15], FLX has been studied by our research group as a positive modulator of mitochondrial function and oxidative metabolism in several tissues [16-19] , including the BAT [20]. Based on the report of Silva et al., 2015 that found increased BAT mitochondrial oxygen consumption, UCP1 expression and higher energy expenditure after neonatal FLX-treatment in male *Wistar* rats [20], we were intrigued by the fact that this treatment could result in a similar response when conducted in an overweight/obesity model.

Recently we demonstrated that FLX treatment in young adult overfed rats reduced weight gain and switched the white and brown fat tissue profile towards to a lean phenotype (resist weight gain). Additionally, they showed improved serum metabolic parameters, decreased food consumption and upregulation of hypothalamic satiety circuitry of the 5-HT system [21]. In terms of energy balance though, white adipose tissue in these overfed rats treated with FLX is likely to be more prone to switch their typical morphology by cellular differentiation. Thus, browning agents seem to be crucial in this process, that by food, drug and genetic manipulation are induced to increase transcriptional profile in white adipocytes that in turns acquire brown fat-like characteristics. Among molecular targets that coordinate browning process are sirtuin 1 (*Sirt1*), an energy sensor activated by an oxidized cellular state with deacetylase activities; peroxisome activated receptor gamma coactivator 1 alpha (*Pgc1α*), master regulator of energy metabolism and mitochondrial biogenesis; PR/SET domain 16 (*Prdm16*) specifically devoted to activate differentiation of subcutaneous fat depots and proliferative program by transcriptional activation, and uncoupling protein 1 (*Ucp1*), marker of the full browning process and thermogenesis [22-24]. Therefore, conducted by our previous finding and the potential novelty of this area, this study was devoted to investigate whether a chronic 5-HT reuptake inhibitor (i.e. Fluoxetine-FLX) exposure in young adult rats postnatally overfed would effectively modulate iBAT mitochondrial function and transcriptional factors associated with browning process in WAT.

2. Methods

2.1 Animals

Twelve female *Wistar* rats of 80-day-old (150-200g) were mated with six male rats (120 days of age, 200-250g) (2 female:1 male) and maintained at room temperature (23±1ºC) in a 12-h light-dark cycle (light 11:00 p.m – 11 a.m.) with free access to water and commercial chow (Labina- Presence®) during pregnancy and suckling period [21]. Twenty-four hours after birth, male rats (6-8g) were assigned randomly in 9 pups

per dam. At postnatal day 3 (PND3), groups were divided by adjustment of litter size: Normofed group remained with 9 pups per litter (total of 3 litters) and overfed group was adjusted to 3 pups per litter (total of 9 litters). Reduced litter size increases food supply to the offspring as previously described by Plagemann [25, 26] and published by our research group [4, 21, 27]. At PND21 (weaning), all male pups were housed in cages with 9 animals/cage (n=27 for both groups) and during the pharmacological treatment until experimental age (PND60) animals were assigned into 3 animals/cage. They received water and commercial chow *ad libitum* until 60 days of age. The present study was approved for Local Ethics Committee for Animal Research of the Federal University of Pernambuco (Protocol number: 0027/2017) and followed the guidelines of laboratory animal care by National Institutes of Health (NIH, Bethesda, USA).

2.2 Pharmacological treatment and experimental groups

From PND39 through PND59, animals received a daily subcutaneous injection of vehicle solution (NaCl: 0.9%, 10ml/kg b.w.) or fluoxetine, a serotonin reuptake inhibitor (SSRI) (10mg/kg b.w., in vehicle solution, 10ml/kg b.w.). According to this pharmacological treatment, rats were assigned in four groups with similar body weight and weight gain since the beginning of experimental model: Normofed + vehicle solution (NV group, n=13), Normofed + fluoxetine (NF group, n=14), Overfed + vehicle solution (OV group, n=13) and Overfed + fluoxetine (OF group, n=14). The pharmacological treatment was administered always in the second hour after the dark cycle starts, to avoid a possible influence of circadian rhythm [20, 21].

2.3 Body weight and Lee Index measurements

Body weight (g) was measured during suckling period (at PND7, 14 and 21), at PND30 and during pharmacological treatment (PND39, 49 and 59) using a digital balance with accuracy of 1 gram [20, 21]. The naso-anal length (mm) was taken at 59 days of age using a graph paper (0.1 mm). To determine Lee index the following equation was adopted: Lee index = cube root of body weight (g)/naso-anal length (cm) x 1000 [28, 29]

2.4 Tissue collection and experimental outcomes

At 60 days of age, rats were euthanized quickly by decapitation and interscapular brown adipose tissue (iBAT), retroperitoneal (rtWAT) and epididymal white adipose tissue (eWAT) were dissected and used freshly for mitochondrial assays (iBAT) or stored at -20 ºC for western blot (iBAT) and Real-time polymerase chain reaction-RT-PCR (eWAT and rtWAT) [20, 21].

2.5 Mitochondria preparation and oxygen consumption

After dissection and homogenization using a Potter-Elvehjem pestle and a digital homogenizer (IKA® RW 20, Germany), samples of iBAT were submitted to differential centrifugation as previously described to obtain mitochondrial portion [20] and quickly quantified spectrophotometrically for protein concentration by Bradford protocol [30]. Mitochondrial analyses were performed at 0.5 mg/mL of final concentration using proper respiration buffer [20]. Mitochondrial respiration was performed in a 600-SL chamber with controlled temperature (28 ºC) connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK) recording basal state (succinate 5mM + rotenone 4µM) and CCCP-induced uncoupling state (1 µM CCCP). In order to verify the contribution of UCP1 in mitochondrial respiration, we also performed the oxygen consumption in the presence of GDP (UCP inhibitor, 1mM). Data were expressed as nmol of oxygen/min/mg of protein.

2.6 Mitochondrial reactive species (RS) production

To measure the mitochondrial RS production, mitochondrial suspensions were incubated with 1 µM of fluorescent probe DCFDA (5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) at 28 ºC and monitored for 5 min in a spectrofluorimeter (OMEGA, USA) at 485 nm excitation and 530 nm emission. The rate of RS production is directly proportional to the increase of fluorescence intensity, estimated by slop from the area under the curve [20].

2.7 Mitochondrial uncoupling protein 1 (UCP1) expression

Western blot (WB) analysis was performed as described previously [20, 31]. iBAT protein amount was verified by Bradford assay [30] and separated by electrophoresis in a 12% SDS-PAGE. Then, proteins were transferred to a PVDF membrane (Amersham Hybond-P PVDF Transfer Membrane GE Healthcare). Membranes were incubated with the primary antibody against UCP 1 (SCBT: SC-6528) and with the secondary antibody (rabbit anti-goat IgG-HRP, SCBT: SC2768). Detection was performed by luminescence reaction using ECL solution (ECL Western blotting system) and exposed to X-ray film. Band intensities were measured using the Image J software (NIH, Maryland, USA) and expressed as arbitrary units. Gel transfer efficiency in equal load was verified using reversible Ponceau staining [20, 31].

2.8 RNA extraction and RT-PCR

Total RNA was extracted from iBAT, rtWAT and eWAT using TRIZOL reagent (Invitrogen, USA) and guanidine isothiocyanate/phenol method [32] for mRNA expression of the following target genes: *Ucp1* and *leptin* in the iBAT, *leptin* in the rtWAT and *Sirt1, Pgc1α, Prdm16* and *Ucp1* in the eWAT. After determination of RNA concentration with Nanodrop (Thermo Scientific, USA) RNA purity was assessed by 260/280 ratio (>1.8). RT-PCR was performed using Rotor-Gene Q (Qiagen, USA) as previously described [21] running three samples by group in duplicate. Cycle threshold (CT) of each targeted gene (detailed information in Table 1) was compared with CT of internal control for each tissue (β-actin for iBAT and β2M for rtWAT and eWAT) and mRNA content was normalized by $2^{-\Delta\Delta Ct}$ formula [33].

2.9 Functional UCP1 evaluation

Moreover, functional UCP1 protein was presented as a recruitment measurement of the uncoupling protein expression (the relevant thermogenesis parameter) taking into account the total amount of mitochondrial protein (quantitatively important in the final mitochondrial activity). Thus, the UCP1 protein levels/mg of protein and total protein amount of mitochondrial homogenate generated the functional UCP1 score, based on the previous publication of Nedergaard and Cannon [34].

2.10 Tissue preparation for oxidative balance evaluation

iBAT was homogenized using TRIS buffer (50mM, pH 7.4, with 1mM EDTA, 1mM sodium orthovanadate and 100 µg/mL PMSF) and an Ultra-Turraz homogenizer (model T10 BS32; IKA, Germany) followed by centrifugation at 461g during 10 min at 4ºC [17]. The supernatant was quantified for protein concentration [30] and further analyses were performed with 300 µg of protein.

2.11 Malondialdehyde concentration

To evaluate lipid peroxidation, malondialdehyde (MDA) levels were assayed by Buege and Aust protocol [35]. The pink pigment resulted by mixing samples with 0.73% thiobarbituric acid (w/v) in equal volumes and boiling for 15 min was measured in a spectrophotometer at 535 nm. Results were expressed as mmol of MDA/mg of protein.

2.12 Carbonyl content measurement

Protein oxidation was performed as described by Levine *et al*, 1990 [36]. In the final process samples were mixed with 6M guanidine hydrochloride and incubated at RT for 30 min. The content was then measured in a spectrophotometer at 370 nm and results were expressed as mmol of Carbonyl/mg of protein.

2.13 Reduced and oxidized glutathione content and REDOX state

GSH and GSSG content were evaluated according to Hissin and Hilf, 1976 [37]. Both glutathiones content was assessed by incubation of samples with 1 mg/mL O-Phthalaldehyde (OPT) at RT. However, for GSSG content samples were prior incubated with 0.04M N-ethylmaleimide for 30 min in RT. Measurement was performed fluorometrically at 350 nm and 420 nm, excitation and emission wavelengths respectively and compared to a known standard GSH and GSSG curves. Data were expressed as µmol/mg of protein. REDOX state was presented as the ratio between GSH and GSSG content.

2.14 Global oxidative status (Oxy-score)

As published before [38, 39], Oxy-score provides an estimative of oxidative damage and was performed by the subtraction between the sum of standardized values of antioxidant (evaluated here by GSH and REDOX state) and oxidative biomarkers (given as MDA, Carbonyls and GSSG content), or: Oxy-score = (ANTIOX – OXY). Data were expressed as arbitrary units and a positive oxy-score indicates prevalence of antioxidant capacity while negative scores suggest an oxidative damage [39].

2.15 Statistics

Data were first tested to normality through Kolmogorov-Smirnov analysis and once proved, two-way ANOVA test followed by Tukey's post hoc test were used to assess differences among groups. Data were expressed as percentage from control group and considered statistically significant at $p<0.05$. All analysis was performed using GraphPad Prism 6.0® software (GraphPad Prism Software, Inc., La Jolla, CA, USA).

3. Results

3.1 Body weight, leptin and Ucp1 expression

Overfed group (black squares, n=9) showed a 37% increase in body weight at day 14 (*p=0.0006), 28% at day 21 (*p<0.0001) and 35% at day 30 (*p<0.0001) compared to normofed group (white circles, n=9). During pharmacological treatment both overfed groups remained heavier than their normofed controls:

OV *vs* NV - 18% at 39 days (*p=0.0037) and 49 days (*p<0.0001), 19% at 59 days (*p<0.0001); OF (blue squares) *vs* NF (gray circles) – 25% at 39 days (†p<0.0001), 22% at 49 days (†p<0.0001) and 18% at 59 days (†p<0.0001). OF group though, decreased body weight compared to OV group after 10 days of treatment: 8% at 49 days ($^{\#}$p=0.0481) and 10% at 59 days ($^{\#}$p=0.0004) (Fig 1A). In Fig. 1B, we showed that OV group had increased Lee index at 59 days of age compared to NV group (19%, n=7, p<0.0001) and OF group showed a discreet reduction in Lee index compared to OV group (6%, n=8, p=0.0224) (Fig 1B). In rtWAT, *leptin* expression had a 2.7-fold increase in the OV group compared to NV group (p<0.0001, n=5). However, high levels were found in NF group compared to NV group (97%, p=0.0017, n=5) while OF group showed reduced expression compared to OV group (55%, p<0.0001, n=5). iBAT *leptin* expression decreased in OV group compared to NV group (80%, p=0.0282, n=4) and increased in OF group compared to OV group (4-fold, p=0.0282, n=5) (Fig 1C). Assessing *Ucp1* expression in iBAT we found an increase in OV group (125%, n=4, p=0.0321) compared to NV group and decrease in OF group compared to OV group (65%, n=4, p=0.0133) (Fig 1C, insert).

3.2 Mitochondrial function of iBAT

UCP1 protein expression was increased only in NF group compared to NV group (70%, n=5, p=0.0076) (Fig 2A). The functional UCP1 protein significantly increased in OF group compared to OV group (56%, p=0.0077, n=5) (Fig 2B). Related to mitochondrial oxygen consumption, in the basal state only OF group presented a higher oxygen consumption compared to OV group (38%, n=6, p=0.0149). In the absence of UCP activity (+GDP), NV group, as expected, decreased basal oxygen consumption in comparison to the intact respiration (-GDP) (43%, n=5, p=0.0027) and both FLX-treated groups increased the basal respiration compared to their controls (85% in NF *vs* NV group, n=5, p=0.0014 and 66% in OF *vs* OV group, n=6, p=0.0035) (Fig 2C). In the uncoupling state, OV group decreased oxygen consumption compared to NV group (35%, n=6, p=0.0440) and both FLX-treated groups exhibited higher oxygen consumption compared to their controls: NF *vs* NV (37%, n=5, p=0.0329) and OF *vs* OV (110%, n=6, p<0.0001). By adding GDP, the mitochondrial uncoupling remained higher in NF group compared to NV group (83%, n=5, p=0.0015) and had an 1.5-fold-increase in OF *vs* OV group (n=6, p=0.0035). Comparing respiration with and without GDP, only OF group showed an increase in uncoupling state post-UCP inhibition (47%, n=6, p=0.0010) (Fig 2D).

3.3 Oxidative stress biomarkers in iBAT

Mitochondrial RS production increased in NF group (108%, n=5, p=0.0010) and in OV group (82%, n=5, p=0.0143) compared to NV group, and reduced in OF group compared with OV group (54%, n=5,

p=0.0036) and with NF group (59%, n=5, p=0.0002) (Fig 3A). MDA concentration increased in NF group (84%, n=5, p=0.0177) and OV group (135%, n=6, p=0.0001) compared to NV group, and decreased in OF group compared to OV group (28%, n=6, p=0.0418) (Fig 3B). Carbonyl content was increased in NF group (67%, n=6, p=0.0072) and OV group (59%, n=5, p=0.0247) compared to NV group (Fig 3C). GSSG content increased in NF group in comparison to NV group (76%, n=5, p<0.0001), and decreased in OF group (50%, n=5, p<0.0001) compared to NF group (Fig 3E). REDOX state was decreased in NF group compared to NV group (62%, n=5, p=0.0092) and increased in OF group compared to NF group (196%, n=5, p=0.0031). There was a tendency to increase REDOX state in OF group when compared to OV group (59%, n=4, p=0.066). Oxy-score measurement showed an 11-fold decrease of NF group compared to NV group (p<0.0001, n=6). OV group also had similar down regulation compared to NV group (12-fold, p<0.0001, n=6). OF group however increased the global OXY-SCORE compared to NF group (8-fold, p<0.0001, n=6) and OV group (9-fold, p<0.0001, n=6).

3.4 Regulation of browning agents in eWAT

Regarding of molecular pathways involved in browning process of eWAT (Fig 4), *Sirt1* expression was decreased in OV group compared to NV group (81%, p=0.0005, n=4) and in OF group compared to NF group (60%, p=0.0001, n=4). Furthermore, there was a trend towards to a significant difference in NF group compared to NV group (36% increase, p=0.08, n=4) and in OF group compared to OV group (~2-fold increase, p=0.071, n=4). The *Pgc1α* expression had a massive increase in both overfed groups, with a 5-fold increase in OV group compared to NV group (p<0.0001, n=4) and a 6-fold increase in OF group compared to NF group (p<0.0001, n=4). OF group, however, showed a 20% increase in *Pgc1α* expression compared to OV group (p=0.0293, n=4). *Prdm16* was highly expressed in OF group compared to NF group (674%, p=0.0063, n=4) and with OV group (235%, p=0.0259, n=4). The expression of *Ucp1* was only significant in OF group compared to NF group (12-fold increase, p=0.0032, n=4), although a trend towards a group difference was found in NF group compared to NV group (88% reduced, p=0.062, n=4) and in OF group compared to OV group (105% increased, p=0.064, n=4).

4. Discussion

Previous studies from our research group reported that the chronic serotonin reuptake inhibition improved BAT mitochondrial function in lean young adult animals when the treatment was conducted during the suckling period [20]. The same treatment was found to be increased serotonin concentration in hypothalamus of 21- and 40-day old rats [40]. Also iBAT amount and energy balance neurocircuitry in overfed animals treated later in life [21] resulting in hypophagia. Such interesting data suggested an

improvement in metabolic efficiency and conducted us to investigate the hypothesis that chronic serotonin modulation in young adult overfed rats would induce a thermogenic and anti-oxidative profile of iBAT.

As expected, overfed animals increased body weight during the suckling period and life course with a higher Lee index at the end of the study. Also, chronic 5-HT reuptake inhibition is related to a lesser weight gain effectively observed in both FLX-treated groups [13, 41, 42]. Previous findings of our research group have pointed out the significant contribution of the central 5-HT system in improving body composition in overfed rats [21] and to better comprehend body weight management in overfed rats we further estimated the expression of the anorexigenic hormone leptin, which is known to be upregulated proportionally to adipose tissue content [43]. We showed earlier that OV and OF group presented inverse white adipose tissue amounts [21] and *leptin* expression herein matches with this finding. However, despite NF animals had previously shown lower WAT content [21] its *leptin* expression was almost twice as higher than NV group. This suggests that 5-HT-derived anorectic response exhibit distinct effects that may rely on early nutritional status, favoring peripheral leptin sensitivity in normofed animals for alternatively energy expenditure. Leptin in lean rodents is a sensor of fat storage regulated by negative feedback loops. In this case maintenance of fat storage paradoxically represents its major physiologically relevant regulator, rather than overfeeding, prone to survival [44].

In iBAT, our data demonstrated an inverse response of *leptin* expression compared to rtWAT. In this case, the singular characteristic of BAT is consistent with its thermogenic activity where increased *leptin* expression is related to multilocular cell morphology, characteristic of vast mitochondrial content. Reciprocally regulation of energy balance genes expression (i.e. *leptin* and *Ucp1*) was already mentioned before [45] and suggests that leptin role relies on adipocyte characteristics, wherein brown adipocytes is likely associated with *Ucp1* downregulation [46]. Overfed animals though presented oppose leptin expression, mainly attributed to the different iBAT content and functionality observed in OV and OF groups. Besides that, is likely that by the timing of gene expression, *Ucp1* was no longer required for thermogenesis in OF group.

UCP1 is devoted to producing heat by uncoupling phosphorylation from energy production in BAT and this has a clinical relevance in obesity therapeutics [47]. The role of UCP1 in thermogenesis was observed here by increased UCP1 expression in the NF group. Da Siva *et al*, 2015 have found at 60 days of age increased UCP1 protein expression in lean animals after neonatal serotonin reuptake inhibition [20]. Our similar result in the normofed group suggests that 5-HTergic effects on UCP1 expression in iBAT are independent of the period in which animals received such pharmacological intervention and plays a crucial role in energy expenditure. Importantly, UCP3 expression in skeletal muscle and its co-existence in BAT is also correlated to energy expenditure [48, 49], although not evaluated in our study. Evidences have emerged to confirm that early overfeeding is associated with iBAT thermogenic hypoactivity and downregulation of

Ucp1 [11, 50]. Also, other reports showed that diet-induced obesity is associated with peripheral serotonin inhibition and reduced BAT thermogenesis [12] reinforcing the role of the central serotonergic system in overfed animals herein demonstrated. Interestingly, OF group did not differ from OV group in UCP1 expression but analyzing mitochondrial uncoupling capacity we observed that this measurement was similarly boosted in both FLX-treated groups.

To the best of our knowledge, this is the first study to evaluate the effects of a chronic serotonin reuptake inhibition in the iBAT mitochondria with this overfeeding model. In this sense, we demonstrated that overfed animals display a lower mitochondrial uncoupling ability suggesting more susceptibility to a metabolic disarrangement and compromising thermoregulation. Besides, impairment of BAT mitochondrial activity could be developed even in the absence of UCP 1 expression due to lack of SIRT3 and its acetylation effect upon UCP1 [51]. This impairment could be related to oxidative stress, that was in fact, demonstrated here. Previously, BAT mitochondria from 28-week-old obese mice showed increased oxygen consumption through complex III and UCP1 upregulation [9]. Such contrary findings may be due to obesity model (i.e. high-fat diet) and time of insult (i.e. 20 weeks). Even with this controversial data, they reported increased oxidative damage and high levels of inflammation. Moreover, mitochondrial oxygen consumption suggests that full innate activity of UCP was improved in both FLX-treated groups. Particularly interesting, our data showed efficient mitochondrial respiration in OF group in both states albeit indistinct UCP1 protein expression. Since we previously observed a positive modulation of iBAT amount in OF group we were wonder whether UCP1 was functionally modulated along with the iBAT phenotype modification described earlier [21]. To address this issue, we assessed one of the physiologically relevant parameter to estimate the full capacity of UCP1 from iBAT, the total UCP1 amount. Such result indicates that a chronic serotonergic manipulation in overfed animals enables functional expression of UCP1 toward an improved mitochondrial activity. This was not observed in NF group suggesting that later 5-HTergic manipulation in juvenile lean animals is not capable of inducing efficient UCP1 activation. As seen, no GDP sensitivity was observed in the uncoupling respiration in NF group although OF group indeed demonstrated higher proton leak when UCP was inhibited. This data may support the thermogenic effect of fluoxetine mediated by improved mitochondrial uncoupling of brown fat in overfeeding conditions, an interesting aspect that has not been demonstrated before.

Mitochondrial reactive species (RS) production was higher in OV and NF groups followed by increased oxidative stress biomarkers. RS production and oxidative stress have been associated with modulation of UCP1 expression in both brown and white adipocytes [52]. There is an emerging fact that increased RS production and thiol REDOX status signals support the mitochondrial uncoupling and non-shivering thermogenesis [53-55]. Both conditions were present in NF animals and associated with oxidative stress, which by signal transduction lipid peroxidation products could induce mitochondrial uncoupling by

activation of UCPs, regulates the mitochondrial membrane potential and excessive ROS damage [56, 57]. Data from Zhang et al., (2016) follow the rationale of nutrient induced-metabolic adjustments, once, by the contrary of what we found in OV group, they report decreased mRNA UCP1 and MDA content in striped hamsters (common Asiatic rodents) restricted for 28 days from their standard rodent chow (80% less than the average food consumption) [58]. In addition, regardless of differences in GSH content, GSSG increase induced a deficient REDOX state suggesting that non-enzymatic antioxidant defense was unfavorable in NF group and a pro-oxidant environment has been established. The disruption of this redox control (i.e. reduced GSH/GSSH system) is implicated in irreversible drug-induced glutathionylation of proteins becoming toxic for cell and may increase the risk of metabolic disease onset in lean animals [59]. Although OV group did not differ from their controls in glutathione measurements, global oxy-score revealed a negative cellular status. The similar results of NF and OV group suggest that both insults (i.e. pharmacological treatment in lean animals and neonatal overfeeding) may be detrimental to the cellular oxidative balance of iBAT later in life. Importantly, glutathione levels have scarce reports in literature when focused on brown adipose tissue. Herein, OF group not only displayed lower GSSG levels and a tendency to improve REDOX status ($p=0.066$) as remarkably improved global oxidative score, indicating a prevalence of antioxidant capacity and lower oxidative damage. As the majority of oxidative stress analyses in OF group were positive modulated, we believe that the outstanding oxidative benefits of the serotonergic manipulation may have contributed to counteract redox impairments of rats that underwent to early nutritional insult.

Until so far, we have attributed the improvement of mitochondrial and cellular oxidative functions after 5-HT manipulation in overfed rats to an efficient brown adipose tissue function. Because non-classic BAT sites can also be targeted for cellular differentiation, we next evaluate whether pharmacological treatment could also switch the transcriptional profile of epididymal white adipose tissue (eWAT), well known to be a potential target, among other adipocyte types, to differentiate into beige/brown fat according to stimuli [13, 60]. In this sense, the OV group showed decreased expression of *Sirt1* and increased expression of *Pgc1α*. *Sirt1* was found to be decreased in adipose tissue of obese humans [61, 62] and rats [63] and its involvement in adipogenesis and inflammation has been discussed over the years with significant attempting to its direct reduced adiponectin correlation due to obesity [63]. Upregulation of *Pgc1α*, one of the well-known transcriptional coactivators family members that regulate several biological processes could be related to impaired energy/lipid metabolism with triacylglycerol accumulation and oxidative stress in eWAT (higher in visceral rather than subcutaneous WAT) [64]. Increased PGC1α reports were already described in obesity models in particular by Dias et al, (2018) that evaluate this transcriptional coactivator in adipose-derived mesenchymal stem cells of 90-day-old rats with a similar overfeeding model of our study [64, 65].

In agreement with our hypothesis, chronic 5-HT reuptake inhibition display an important remodeling role in browning process of eWAT in overfed rats, with overexpression of the majority of genes analyzed and tendency towards an increase in *Sirt1* (p=0.070). *Sirt1* expression in adipose tissue may decrease fat storage, promote lipolysis and protect against obesity-induced inflammation [66] and its deacetylation effect upon *Pgc1α* drives specific molecular differentiation of WAT through brown fat-like differentiation [67]. This was reinforced by overexpression of *Prdm16* that may induces progenitor cell differentiation into brown-like pre-adipocytes with final activation of *Ucp1*, suggesting that at least at the transcriptional level the full browning process was effectively acquired in FLX-treated overfed animals, an outstanding effect reported here for the first time.

Taken together our results indicated that FLX treatment later in life prevents body weight gain of overfed rats partially through oxidative improvement in iBAT and molecular differentiation towards a beige/brown phenotype, while for normofed animals the classic drug induced-body weight loss is detrimental, at least in the point of view of oxidative balance.

5. Conclusions

Serotonergic reuptake inhibition in young adult overfed rats restores iBAT mitochondrial activity, REDOX function and crucially drives adaptation of white fat towards browning adipogenesis. Our integrative comprehension of energy balance regulation in overweight/obesity is sustained by this and previous data reinforcing mitochondrial role in obesity and covering the understanding of serotonergic system modulation in such a broad pandemic disease.

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Table 1: Specifications of target genes

Gene	Sequence	Annealing temperature	Tissue analyzed
Beta -2 microglobulin (β2M)	Sense: TGA CCG TGA TCT TTC TGG TG Antisense: ACT TGA ATT TGG GGA GTT TTC TG	48 °C	eWAT and rtWAT
Beta-actin (β-actin)	Sense: CTA TCG GCA ATG AGC GGT TCC Antisense: GCA CTG TGT TGG CAT AGA GGT	53 °C	iBAT
Leptin	Sense: CAG GCT CTC TGG CTT CTG Antisense: GAG ACC TCC TCC ATC TGC TG	50 °C	rtWAT and iBAT
Peroxisome activated receptor gamma coactivator 1 alpha (Pgc1a)	Sense: AAC AGC AAA AGC CAC AAA GA Antisense: AAG TTG TTG GTT TGG CTT GA	48 °C	eWAT
PR/SET domain 16 (Prdm16)	Sense: ACG GAA GAT GGA AAT CGG GG Antisense: GAA CTT CTC GCT GCC CAA AC	50 °C	eWAT
Sirtuin 1 (Sirt1)	Sense: CAC AGC AAG GCG AGC ATA AA Antisense: GGC AGA CAA TTT AAT GGG GTG AA	49 °C	eWAT
Uncoupling protein 1 (Ucp1)	Sense: CTC CAC AAA TAG CCC TGG TG Antisense: GGT GAT GAT GTC TGC TAG GC	49 °C	iBAT and eWAT

Figure captions

Figure 1: Body weight (g) measurements (A), *leptin* and *Ucp 1* expression (B) in retroperitoneal white adipose tissue (rWAT) and interscapular brown adipose tissue (iBAT) of male *wistar* rats from NV, NF, OV and OF groups. (A) Body weight at 7, 14, 21 and 30 days of age and during pharmacological treatment (39, 49 and 59 days of age) (*p<0.05 between OV and NV groups, #p<0.05 between OF and OV groups, †p<0.05 between OF and NF groups, n=9 per group). (B) Lee index at 59 days of age (n=7-8 per group), (C) mRNA levels of leptin in rtWAT and iBAT at 60 days (n=4-5 per group) and (C-insert) *Ucp1* gene expression in iBAT at 60 days (n=4-5 per group). Data presented as mean±SEM, *p≤0.05, **p≤0,01, ***p≤0.001, ****p≤0.0001. All data were compared by two-way ANOVA with Tukey's test for multiple comparisons. White: normofed + vehicle group; Gray: normofed + fluoxetine group; Black: overfed + vehicle group and Blue: overfed + fluoxetine group.

Figure 2: Mitochondrial oxidative capacity of interscapular brown adipose tissue (iBAT) of male *wistar* rats from NV, NF, OV and OF groups at 60 days of age. (A) UCP1 protein expression, (B) total UCP1 protein levels (Based on UCP1 protein expression and total protein concentration of mitochondria, n=5) (C) mitochondrial oxygen consumption in basal state and (D) in CCCP-induced uncoupling state (both performed with complex II substrates and in the absence or presence of GDP [UCP inhibitor]). Data presented as mean±SEM. All data were compared by two-way ANOVA with Tukey's test for multiple comparisons. *p≤0.05, **p≤0.01, *** p≤0.001, n=5-6 per group. White: normofed + vehicle group; Gray: normofed + fluoxetine group; Black: overfed + vehicle group and Blue: overfed + fluoxetine group.

Figure 3: Oxidative stress measurements of interscapular brown adipose tissue (iBAT) of male *wistar* rats from NV, NF, OV and OF groups at 60 days of age. (A) Mitochondrial RS production, (B) MDA concentration, (C) Carbonyl content, (D) GSH content, (E) GSSG content, (F) REDOX state (Ratio between GSH and GSSH) and (G) Oxy-score (Ratio between antioxidant and pro-oxidant measurements of oxidative stress). Data presented as mean±SEM. Groups were compared by two-way ANOVA with Tukey's test for multiple comparisons. *p≤0.05, **p≤0.01, *** p≤0.001, **** p≤0.0001, n=4-6 per group. White: normofed + vehicle group; Gray: normofed + fluoxetine group; Black: overfed + vehicle group and Blue: overfed + fluoxetine group.

Figure 4: Gene expression of browning agents in epididymal white adipose tissue (eWAT) of male *wistar* rats from NV, NF, OV and OF groups at 60 days of age. Data presented as mean±SEM, Groups

were compared by two-way ANOVA with Tukey's test for multiple comparisons. *p≤0.05, **p≤0.01, *** p≤0.001, **** p≤0.0001, n=4 per group. *Sirt1: sirtuin 1; Pgc1α: peroxisome activated receptor gamma coactivator 1 alpha; Prdm16: PR/SET domain 16; Ucp1: Uncoupling protein 1.* White: normofed + vehicle group; Gray: normofed + fluoxetine group; Black: overfed + vehicle group and Blue: overfed + fluoxetine group.

Highlights

- **Postnatal overfed rats display functional UCP1 after SSRI treatment**
- **5-HT reuptake inhibition induces UCP-independent uncoupling in overfed rats**
- **FLX-treated normofed rats showed increased oxidative stress**
- **FLX drives browning of white fat in overfed but not in normofed rats**

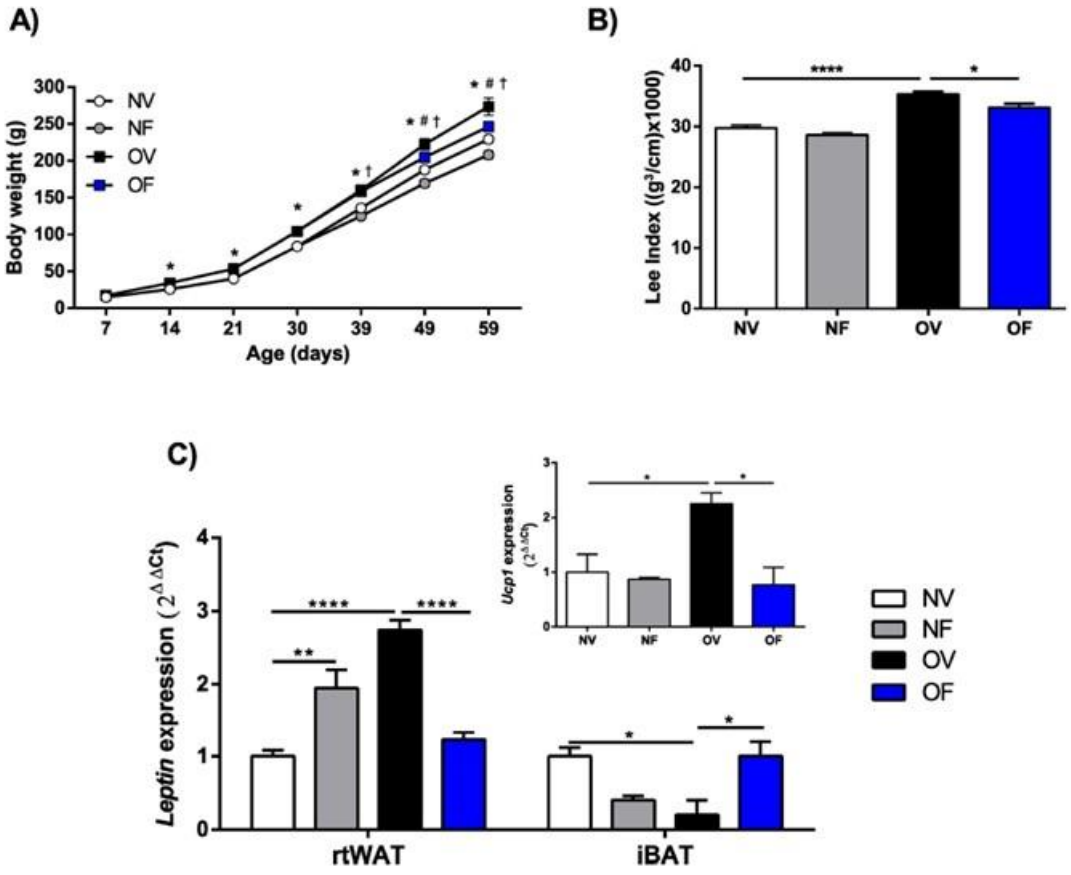
A)
Body weight (g)
NV
NF
OV
OF
Age (days)
B)
Lee Index ((g³/cm)x1000)
C)
Leptin expression (2^-ΔΔCt)
Ucp1 expression
rtWAT
iBAT

Figure 1

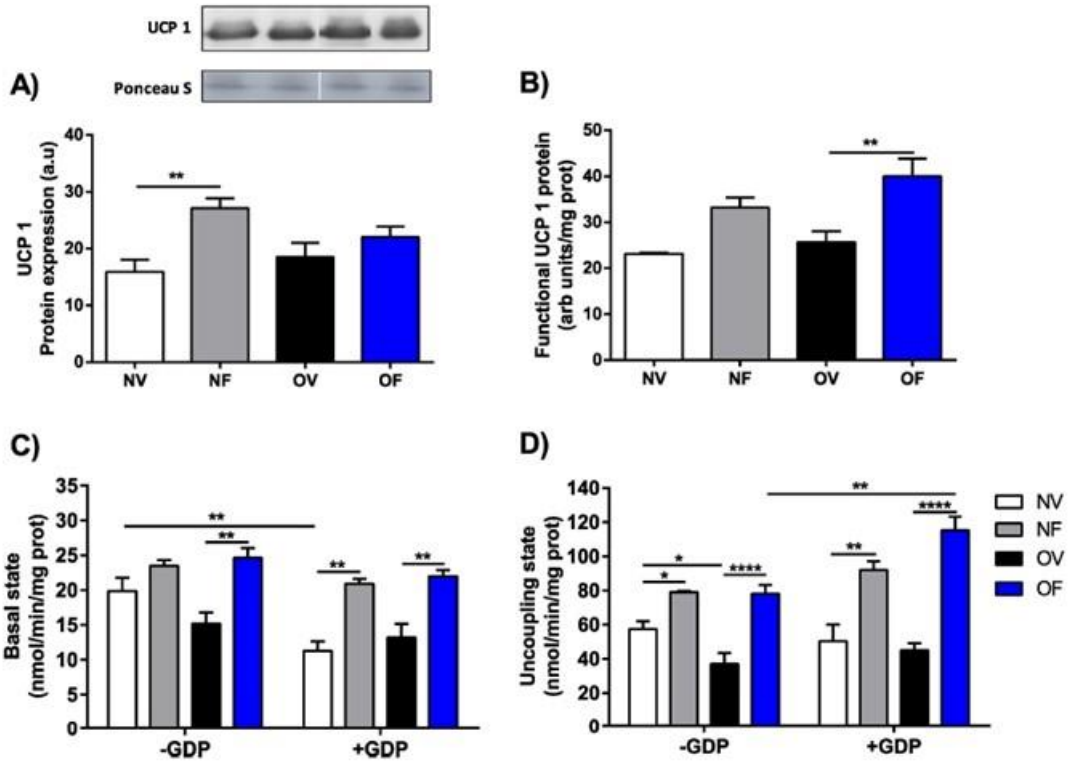
A)
UCP 1
Ponceau S
UCP 1
Protein expression (a.u)
40
30
20
10
0
**
NV
NF
OV
OF
B)
Functional UCP 1 protein
(arb units/mg prot)
50
40
30
20
10
0
**
NV
NF
OV
OF
C)
Basal state
(nmol/min/mg prot)
35
30
25
20
15
10
5
0
**
**
**
**
-GDP
+GDP
D)
Uncoupling state
(nmol/min/mg prot)
140
120
100
80
60
40
20
0
*
*
****
**
**
****
-GDP
+GDP
NV
NF
OV
OF

Figure 2

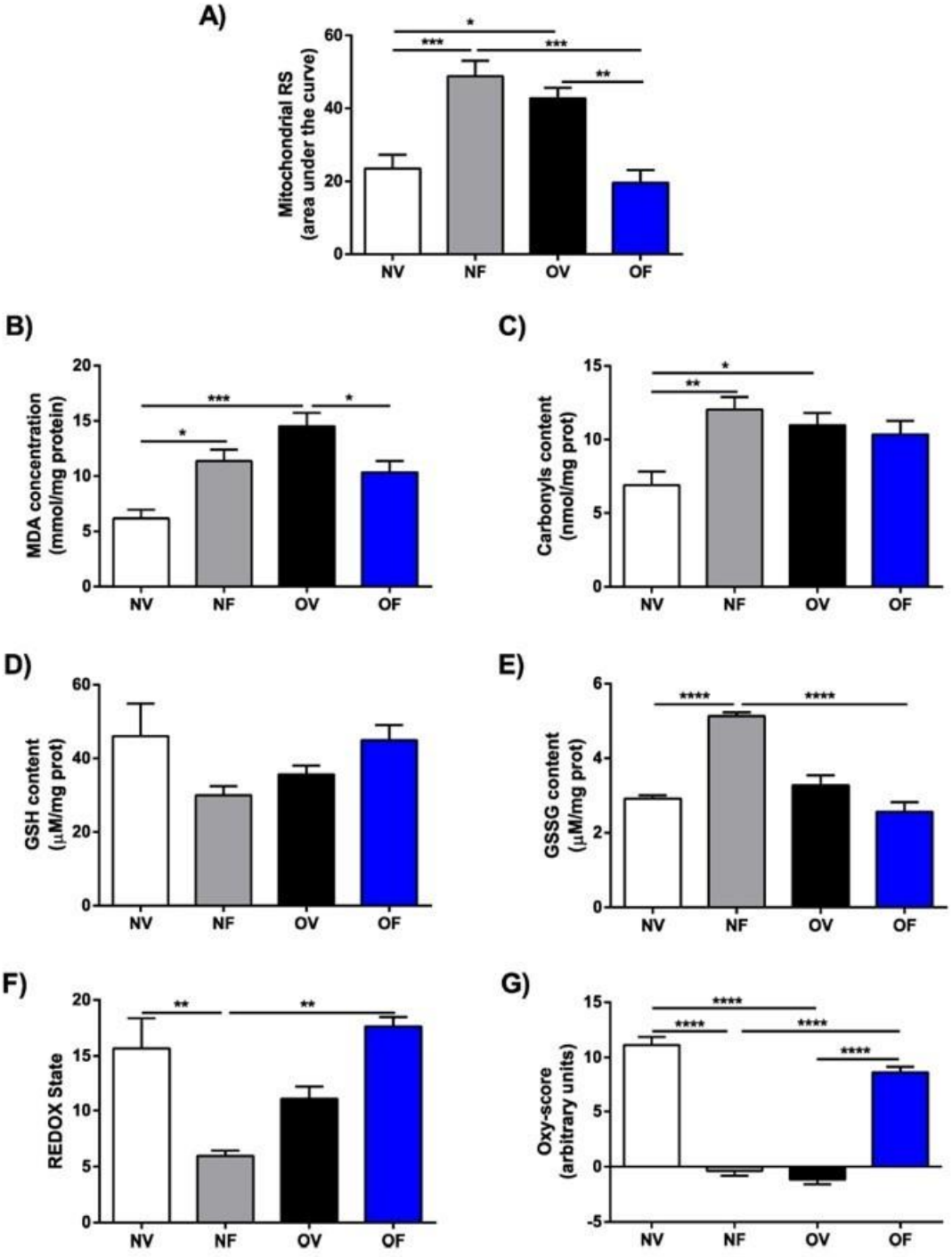
A)
Mitochondrial RS (area under the curve)
60
40
20
0
*
***
***
**
NV
NF
OV
OF
B)
MDA concentration (mmol/mg protein)
20
15
10
5
0
***
*
*
NV
NF
OV
OF
C)
Carbonyls content (nmol/mg prot)
15
10
5
0
*
**
NV
NF
OV
OF
D)
GSH content (µM/mg prot)
60
40
20
0
NV
NF
OV
OF
E)
GSSG content (µM/mg prot)
6
4
2
0
****
****
NV
NF
OV
OF
F)
REDOX State
20
15
10
5
0
**
**
NV
NF
OV
OF
G)
Oxy-score (arbitrary units)
15
10
5
0
-5
****
****
****
****
NV
NF
OV
OF

Figure 3

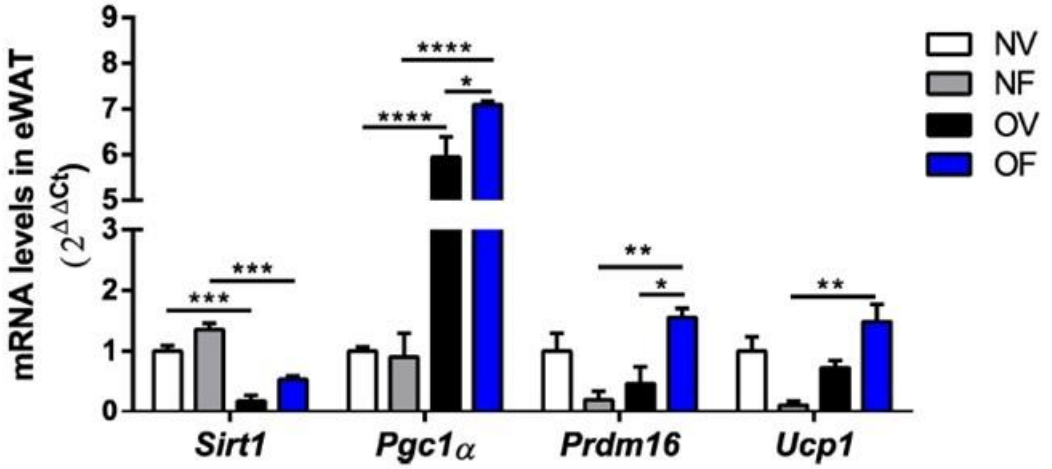
mRNA levels in eWAT
$(2^{\Delta\Delta Ct})$
NV
NF
OV
OF
Sirt1
Pgc1α
Prdm16
Ucp1

Figure 4

6 CONSIDERAÇÕES FINAIS

Diante dos índices cada vez maiores de obesidade, o entendimento dos mecanismos associados ao desenvolvimento/progressão desse distúrbio metabólico bem como do potencial envolvimento de manipulações farmacológicas, como a fluoxetina, se torna clinicamente relevante. O presente modelo experimental estudado proporcionou resultados que elucidam melhor, mas não totalmente, os efeitos da inibição crônica da recaptação de serotonina sobre a bioenergética mitocondrial, balanço oxidativo e controle do balanço energético em condições de sobrepeso/obesidade.

Baseado nos nossos resultados, sugerimos que a administração crônica com fluoxetina atuou revertendo as disfunções mitocondriais e oxidativas encontrados no hipotálamo e tecido adiposo marrom de ratos machos supernutridos, mas não em animais normonutridos. Demonstramos ainda que essa readaptação mitocondrial foi acompanhada pela indução de expressão gênica favorável ao aumento da eficiência metabólica/mitocondrial nos tecidos analisados: aumento de biogênese mitocondrial no hipotálamo e do fenótipo bege no tecido adiposo branco. Em conjunto, esses dados corroboram nossas hipóteses iniciais que sugerem uma forte relação entre a redução do peso corporal em animais supernutridos tratados com fluoxetina com maior participação mitocondrial em termos de metabolismo energético e possível termogênese adaptativa.

Entretanto, ainda se faz necessário um entendimento mais profundo dos mecanismos regulatórios do balanço energético corporal associados a inibição crônica de recaptação de serotonina como a relação entre fármaco e mitocôndria através de receptores serotoninérgicos, a interação entre serotonina e outros neurotransmissores, a participação do sistema nervoso simpático e sistema endócrino na regulação da função mitocondrial e principalmente da extensão dos efeitos da administração farmacológica a longo prazo.

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ANEXO A – COMITÊ DE ÉTICA

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Recife, 08 de dezembro de 2017.

Ofício nº 118/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.ª Claudia Jacques Lagranha**
Núcleo de Educação Física e Ciências do Esporte
Centro Acadêmico de Vitória
Universidade Federal de Pernambuco
Processo *online* nº **0027/2017**

Certificamos que a proposta intitulada **"Avaliação central e periférica da bioenergética mitocondrial e expressão de genes e proteinas chaves no controle do peso corporal de ratos supernutridos tratados com fluoxetina"**, registrada com o nº **0027/2017**, sob a responsabilidade de Prof.ª **Claudia Jacques Lagranha** - 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 DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 06/12/2017.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	08/12/2017 a 31/07/2021
Espécie/linhagem/raça	Ratos heterogênicos Wistar
Nº de animais	96
Peso/Idade	200-250g/120 dias (progenitores machos) 150-200g/80 dias (progenitoras fêmeas) (?)/60 dias (prole machos)
Sexo	Machos (80) e fêmeas (16)
Origem	Biotério do CAV/UFPE

Atenciosamente

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1801584

ANEXO B – COMPROVANTE DE SUBMISSÃO DO ARTIGO 1

Submission Confirmation

Brain Research <eesserver@eesmail.elsevier.com>
Qui, 06/02/2020 14:55
Para: rudafeitoza@hotmail.com <rudafeitoza@hotmail.com>; feitozaruda@gmail.com <feitozaruda@gmail.com>

*** Automated email sent by the system ***

Dear Dr. Braz:

Your submission, Systematic review of serotonin reuptake inhibitors (SSRI) effects on the rat brain mitochondria, article type Research paper, for Brain Research has been received by the Editorial Office and will be processed as soon as possible.

You may log onto EES as an Author to view your submission at any time by entering these login details:

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The Brain Research Editorial Office

ANEXO C – COMPROVANTE DE SUBMISSÃO DO ARTIGO 2

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The European Journal of Pharmacology <eesserver@eesmail.elsevier.com>
Sex, 24/01/2020 16:49
Para: rudafeitoza@hotmail.com <rudafeitoza@hotmail.com>

*** Automated email sent by the system ***

Dear Dr. Glauber Rudá Braz,

You have been listed as a Co-Author of the following submission:

Journal: European Journal of Pharmacology
Title: Serotonin modulation in overfed rats improves hypothalamic mitochondrial respiration, reduces oxidative stress and induces mitochondrial biogenesis
Corresponding Author: Claudia Lagranha
Co-Authors: Glauber Rudá F Braz; Severina Cassia A Silva; Anderson Apolonio S Pedroza; Flavia A de Lima; Maria Daniele de Lemos; Aline Isabel da Silva;

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If you did not co-author this submission, please do not follow the above link but instead contact the Corresponding Author of this submission at lagranha@hotmail.com.

Thank you,

European Journal of Pharmacology

ANEXO D – COMPROVANTE DE ACEITE DE PUBLICAÇÃO DO ARTIGO 3

Date: 01/13/2020
To: "Claudia J Lagranha" lagranha@hotmail.com
From: "Loren E Wold" wold.5@osu.edu
Subject: Your Submission

Ref.: Ms. No. LFS-D-19-04590R1
Chronic serotonin reuptake inhibition uncouples brown fat mitochondria and induces beiging/browning process of white fat in overfed rats
Life Sciences

Dear Professor Lagranha,

We are pleased to inform you that your manuscript has been accepted for publication in Life Sciences.

Your accepted manuscript will now be transferred to our production department and work will begin on creation of the proof. If we need any additional information to create the proof, we will let you know. If not, you will be contacted again in the next few days with a request to approve the proof and to complete a number of online forms that are required for publication.

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Thank you for your contribution to Life Sciences. We look forward to publishing future work from your laboratory.

Sincerely yours,

Loren E. Wold, Ph.D., F.A.H.A., F.A.P.S.
Editor-in-Chief
Life Sciences

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ANEXO E – PUBLICAÇÕES DURANTE O PERÍODO DE DOUTORADO

Behavioural Processes 170 (2020) 103981

Contents lists available at ScienceDirect

Behavioural Processes

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Early weaning disrupts feeding patterns in female juvenile rats through 5HT-system modulations

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ARTICLE INFO

Keywords:
Breastfeeding
Early weaning
Feeding pattern: Food intake
Maternal care
Serotonin

ABSTRACT

Convergent evidence in literature shows that rapid disruption of maternal care and breastfeeding due to an early weaning protocol changes the development of several neurobehavioral patterns in rodents, including the circadian pattern of feeding. The serotonergic system has been associated with the control of feeding patterns. Therefore, we aim to evaluate the patterns of feeding, the mRNA expression of 5HT-1b, 5HT-2c, and SERT on the hypothalamus, brainstem, and the body weight of female juvenile *Wistar* rats, submitted to early (PND15) or regular (PND30) weaning. The results demonstrate that early weaning promotes an increase in food intake in a 24-h period, in the dark phase of the circadian cycle and in the four-hour time intervals at the beginning of the dark and light phases. Also, early weaning decreases the mRNA expression of 5HT-1b, 5HT-2c, and SERT on the hypothalamus, but increases it on the brainstem. Additionally, early weaning promotes an increase in body weight. Therefore, the present data demonstrate that early weaning changes the patterns of feeding in juvenile female rats and suggests that this behavioral modification is due to the modulations promoted in the 5HT-system.

1. Introduction

In the past decades, studies have shown that the 5HT-system plays a key role in modulating rhythmic activities in the whole body (Mendoza et al., 2008; Nakamaru-Ogiso et al., 2012; Paulus and Mintz, 2013). The cell bodies of serotonergic neurons are located in the raphe nuclei at the brainstem. They project and send signals to the central clock regulating the activities of central oscillator genes and, consequently, rhythmic activities (Glass et al., 2003; Meyer-Bernstein et al., 1997; Meyer-Bernstein and Morin, 1996).

From the initial developmental stages of life, evidence shows that rhythmic activity continues and is modulated during postnatal life (Frank et al., 2017). The transition from late pregnancy to lactation seems to be essential for the establishment of a rhythmic activity pattern and this period is associated with changes in expression of both the central and peripheral organs (Casey et al., 2014; Plaut and Casey, 2012).

Maternal care during the postnatal period is a significant factor that helps in adjustment of the pups' rhythmic behavior during the transition from late pregnancy to lactation (Takahashi et al., 1984). In studies carried out with blinded pups by eye enucleation it has been observed that pups' rhythms get dragged when they are crossed to a fostering mother. Eye enucleation allows the rhythms of the animal to be influenced only by the dam and not by the light/dark cycle (Honma et al., 1987; Sugishita et al., 1993). The dam's breast milk, via its compounds' circadian fluctuations, seems to be a significant factor for a pup's rhythm establishment. Leptin, one of the major hormones regulating feeding and satiety, is passed to the pups through the breast milk, which shows a correlated rhythm between the dam's blood and the breast milk levels at PND15 (Nozhenko et al., 2015). In the breast milk of humans, it has been seen that melatonin has fluctuations in its concentrations depending on the phase of the circadian cycle (Kanner et al., 2016). The circadian variation in the tryptophan content in breast milk is correlated to melatonin secretion in a child's urine, which is a determinant of

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Body composition, biochemical, behavioral and molecular alterations in overfed rats after chronic exposure to SSRI

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ARTICLE INFO

Keywords:
Overfeeding
Serotonin
Selective serotonin reuptake inhibitors (SSRIs)
Food behavior
Energy balance
Hypothalamus

ABSTRACT

Serotonin (5-HT) plays a regulatory role in coordinating the neural circuits regulating energy balance, with differences in both 5-HT availability at the synapse and the activity of 5-HT receptors mediating anorectic (*via* POMC/CART activation) and orexigenic (*via* NPY/AgRP activation) responses. In conditions of overweight and obesity the control of energy balance is clearly deregulated, and serotonergic modulation appears to make a significant contribution to weight gain. Fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI) that increases 5-HT availability in the synaptic cleft may thus have potential effects on energy balance. Our aim was to use an overfeeding model to investigate the effects of chronic FLX treatment on energy balance-related parameters regulated by hypothalamic neuropeptides. Nursing male *Wistar* rats were assigned to normofed (9 pups/dam) or overfed (3 pups/dam) groups beginning at 3 days of age and continuing until 21 days of age, when commercial chow and water were made available *ad libitum* until experimental treatments were begun. From 39 through 59 days of age groups were divided according to pharmacological treatment: 1) NV group, normofed + vehicle solution (NaCl 0.9%, 10 ml/kg b.w.), 2) NF group, normofed + FLX (10 mg/kg b.w., in vehicle solution, 10 ml/kg b.w.) 3), OV, overfed + vehicle solution and 4) OF, overfed + FLX. At 60 days of age, body weight, white and brown adipose tissue content, and food intake were determined, and serum biochemical parameters and hypothalamic neuropeptide gene expression were measured. Results showed that FLX induced reductions in several murinometric indices, improvement of adipose profile, hypophagic behavior, reduction in serum parameters, and positive modulation of hypophagia-related genes. These data suggest that the beneficial effects of FLX-treatment on overfeeding-induced physical and behavioral effects in rats was due to hypothalamic alterations that led to improvement in energy balance in animals with a compromised metabolism.

1. Introduction

Obesity affects a large proportion of the world population, and it places those people at a high risk of developing metabolic comorbidities. Early nutritional inadequacies contribute to lifelong disease processes evidenced by the noncommunicable diseases spectrum in which people have faced as a result of, but not exclusive to, unhealthy eating habits earlier in life [1]. Alarmingly, obesity rates are expected to rise at historic levels through 2030 [2] and an obesogenic environment in childhood undoubtedly contributes to those numbers. Estimates for 2016 categorize 41 million children in their first 5 years of age as either overweight or obese [3].

Obesity, by definition, is characterized by an excess caloric intake and reduced energy expenditure that increase adipose tissue deposition and hence body weight [4]. Normal metabolism is thought to be perturbed as long as overweight/obesity persist. As essential integrators of metabolic signals, hypothalamic neuropeptide with orexigenic and anorexigenic functions interpret peripheral neural inputs and generate neural signals controlling energy homeostasis [5]. Specifically in the arcuate nucleus of hypothalamus, activation of pro-opiomelanocortin

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frontiers in Neuroscience

ORIGINAL RESEARCH
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Maternal Protein Restriction in Two Successive Generations Impairs Mitochondrial Electron Coupling in the Progeny's Brainstem of *Wistar* Rats From Both Sexes

*David F. Santana[1], Diorginis S. Ferreira[2], Glauber Ruda F. Braz[1], Shirley M. S. Sousa[1], Tercya Lucidi de Araújo Silva[1], Dayane Aparecida Gomes[1,3], Mariana P. Fernandes[4,5], Belmira Lara Andrade-da-Costa[1,3] and Claudia J. Lagranha[1,5]**

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Santana DF, Ferreira DS, Braz GRF, Sousa SMS, Silva TLdA, Gomes DA, Fernandes MP, Andrade-da-Costa BL and Lagranha CJ (2019) Maternal Protein Restriction in Two Successive Generations Impairs Mitochondrial Electron Coupling in the Progeny's Brainstem of Wistar Rats From Both Sexes. Front. Neurosci. 13:203. doi: 10.3389/fnins.2019.00203

Maternal protein deficiency during the critical development period of the progeny disturbs mitochondrial metabolism in the brainstem, which increases the risk of developing cardiovascular diseases in the first-generation (F1) offspring, but is unknown if this effect persists in the second-generation (F2) offspring. The study tested whether mitochondrial health and oxidative balance will be restored in F2 rats. Male and female rats were divided into six groups according to the diet fed to their mothers throughout gestation and lactation periods. These groups were: (1) normoprotein (NP) and (2) low-protein (LP) rats of the first filial generation (F1-NP and F1-LP, respectively) and (3) NP and (4) LP rats of the second filial generation (F2-NP and F2-LP, respectively). After weaning, all groups received commercial chow and a portion of each group was sacrificed on the 30th day of life for determination of mitochondrial and oxidative parameters. The remaining portion of the F1 group was mated at adulthood and fed an NP or LP diet during the periods of gestation and lactation, to produce progeny belonging to (5) F2R-NP and (6) F2R-LP group, respectively. Our results demonstrated that male F1-LP rats suffered mitochondrial impairment associated with an 89% higher production of reactive species (RS) and 137% higher oxidative stress biomarkers, but that the oxidative stress was blunted in female F1-LP animals despite the antioxidant impairment. In the second generation following F0 malnutrition, brainstem antioxidant defenses were restored in the F2-LP group of both sexes. However, F2R-LP offspring, exposed to LP in the diets of the two preceding generations displayed a RS overproduction with a concomitant decrease in mitochondrial bioenergetics. Our findings demonstrate that nutritional stress during the reproductive life of the mother can negatively affect mitochondrial metabolism and oxidative balance in the brainstem

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Influence of maternal protein malnutrition on oxidative stress and regulators of mitochondrial biogenesis in female rat hearts over succeeding generations

Severina Cássia Andrade Silva[a], Glauber Rudá Feitoza Braz[b], Luciana Caroline Paulino do Nascimento[c], David Filipe Santana[b], Anderson Apolonio da Siva Pedroza[a], Tercya Lucidi Araujo Silva[b], Mariana Pinheiro Fernandes[d], Donald F. Selliti[e], Claudia Jacques Lagranha[a,b,*]

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ARTICLE INFO

Keywords:
Intergenerational
Malnutrition
Mitochondrial biogenesis
Female
Heart

ABSTRACT

Aims: We sought to evaluate the effects of maternal protein restriction (LP) on oxidative balance and transcription factors for mitochondrial biogenesis in the hearts of young female rats of both the first (F1) and second (F2) generation.

Main methods: We evaluated oxidative stress biomarkers (lipid peroxidation and protein oxidation), enzymatic antioxidant defense (activity of superoxide dismutase-SOD, catalase, and glutathione-S-transferase-GST), nonenzymatic antioxidant defense (reduced glutathione-GSH and sulfhydryl groups) and gene expression of AMPK, PGC-1α and TFAM.

Key findings: Interestingly, lipid peroxidation was decreased (49%, $p < 0.001$) in the LP-F1 group and 59% ($p < 0.001$) in LP-F2. In enzymatic defense, we observed increases in SOD activity in the LP-F1 group (79%, $p = 0.036$) and in CAT activity (approximately 40%, $p = 0.041$). GSH was increased in F2 in both groups (LP 546%, $p < 0.0001$ and in NP 491.7%, $p < 0.0001$). With respect to mitochondrial biogenesis gene transcription, we observed a decrease in AMPK (60%, $p < 0.0001$) and an increase in PGC-1α (340%, $p < 0.001$) in LP compared to NP in the F1 generation. TFAM was decreased in LP-F2L compared to NP-F2L (42%, $p = 0.0069$) and increased in LP-F2 compared to LP-F1 (160%, $p = 0.0037$).

Significance: Our study contributes to knowledge of inheritance, showing that despite the potential mitochondrial 'inheritance' of cardiovascular damage caused by maternal malnutrition, that damage is not cross-generational and can be eliminated with proper nutrition in the F1 generation.

1. Introduction

Nutritional deficiency during pregnancy and lactation has been shown to predispose the first generation to development of lifelong metabolic and cardiovascular disease [1,2]. According to WHO, by 2030, nearly 23.6 million people will die from cardiovascular disease (CVD) [3], primarily in developing countries that are most affected by poor maternal diet. Experimental studies on protein restriction have been linked to increased incidence of metabolic disease in the offspring of undernourished mothers and to long-lasting impairments in mitochondrial function [4]. These include increased generation of reactive oxygen species (ROS) in central and peripheral tissues, including the brainstem and heart, as we previously demonstrated in the F1 generation of male rats born to malnourished mothers [5,6]. Oxidative stress, in turn, alters the structure and function of both lipids and proteins [7], which contributes directly to cardiac injury [8]. Our research group has also recently shown that females are less susceptible to oxidative stress induced by maternal low-protein (LP) diet than are males [9], suggesting that in reasonable concentrations, estrogen may act as an antioxidant to reduce the risk of cardiac disease [10]. Its protective effect may be related to the removal of free radicals [11] and to its transcriptional regulation of antioxidant enzymes that contribute to

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RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

Mitochondrial impairment following neonatal overfeeding: A comparison between normal and ischemic-reperfused hearts

Cristiane de Moura Freitas[1] | Luciana Caroline Paulino do Nascimento[1] | Glauber Rudá Feitoza Braz[2] | Severina Cassia Andrade-Silva[1] | Nelson C. Lima-Junior[3] | Tercya de Araujo Silva[2] | Mariana Pinheiro Fernandes[3] | Diorginis José Soares Ferreira[4] | Claudia Jacques Lagranha[1,2,3]

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Abstract

Overweight and obesity are established factors underpin several metabolic impairments, including the cardiovascular. Although the diversity of factors involved in overweight/obesity-induced cardiovascular diseases, mitochondria has been highlighted due to its role in cardiac metabolism. As obesity can be originated in early postnatal life, the current study evaluates the effects of neonatal overfeeding on the cardiac mitochondrial bioenergetics and oxidative balance in rats that underwent an ischemia-reperfusion insult. Seventy-two hours after delivery, Wistar rat litters were randomly assigned into the control (C; nine pups per mother) and the Overfed (OF; three pups per mother) groups throughout the lactation period. At weaning, male offspring were fed with laboratory chow ad libitum until sacrifice at 30 and 60 days of life. Mitochondrial heart bioenergetics and oxidative balance showed to be deeply affected by neonatal overfeeding at both ages. Interestingly, after ischemia-reperfusion insult I/R (Langendorff or mineral oil incubation), most parameters evaluated in OF animals were not influenced by additional ischemic-reperfusion injury. Our findings demonstrated that suckling overfeeding deregulates cardiac mitochondrial alike to ischemia-reperfusion insult by disengaging electrical mitochondrial coupling and potentiate oxidative stress, wherein the neonatal overfeeding shows to be so detrimental as I/R. Our findings support the concept that nutritional insults in the critical development periods increase the risk for cardiovascular disease and mitochondria impairments throughout life while oxidative damage change between molecular targets.

KEYWORDS
cardiovascular disease, ischemia-reperfusion, mitochondria, overfeeding, oxidative stress

Cristiane de Moura Freitas and Luciana Caroline Paulino do Nascimento have contributed equally in this study.

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RESEARCH REPORT

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Serotonin transporter inhibition during neonatal period induces sex-dependent effects on mitochondrial bioenergetics in the rat brainstem

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Abstract

The serotonin reuptake is mainly regulated by the serotonin transporters (SERTs), which are abundantly found in the raphe nuclei, located in the brainstem. Previous studies have shown that dysfunction in the SERT has been associated with several disorders, including depression and cardiovascular diseases. In this manuscript, we aimed to investigate how gender and the treatment with a serotonin selective reuptake inhibitor (SSRI) could affect mitochondrial bioenergetics and oxidative stress in the brainstem of male and female rats. Fluoxetine, our chosen SSRI, was used during the neonatal period (i.e., from postnatal Day 1 to postnatal Day 21—PND1 to PND21) in both male and female animals. Thereafter, experiments were conducted in adult rats (60 days old). Our results demonstrate that, during lactation, fluoxetine treatment modulates the mitochondrial bioenergetics in a sex-dependent manner, such as improving male mitochondrial function and female antioxidant capacity.

KEYWORDS
brain, fluoxetine, oxidative phosphorylation, UCP 2

1 | INTRODUCTION

It is well known that only 2% of total body serotonin (5-hydroxytryptamine; 5-HT) is produced in the central nervous system (Halliday, Baker, & Harper, 1995). However, this neurotransmitter has an essential role in several functions, including neurogenesis, neuronal differentiation, synaptogenesis, stress regulation, anxiety, food intake and cardiorespiratory functions (Heisler, Zhou, Bajwa, Hsu, & Tecott, 2007; Lam & Heisler, 2007; Lam et al., 2008; Rapport, Green, & Page, 1948; Whitaker-Azmitia, 2001; Whitaker-Azmitia, Druse, Walker, & Lauder, 1996). The majority, if not the total amount, of serotonin produced in the central nervous system comes from neurons in the raphe nuclei located inside of the brainstem (Henderson, Keay, & Bandler, 2000). Characterized as a core structure involved in vital networks for life, the brainstem regulates a plenty of homeostatic activities required for survival and environmental adaptations (Nicholls & Paton, 2009).

Despite the complexity in the networks within this structure, the autonomic modulation-controlled cardiorespiratory function has been extensively described, and it mainly relies on neurons that reside in pontomedullary regions, rostral and caudal ventrolateral medulla (RVLM and CVLM, respectively) and nucleus of the solitary tract (NTS; Guyenet, 2006;

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RESEARCH ARTICLE

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Neonatal treatment with fluoxetine improves mitochondrial respiration and reduces oxidative stress in liver of adult rats

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Abstract

Recent studies have shown that exposure to fluoxetine treatment induces excessive production of ROS, and alters the antioxidant defense system in various tissues and cell types, mainly the liver. When fluoxetine is administered intraperitoneally, the drug rapidly reaches high concentrations in the liver, has potentially multiple toxic effects on energy metabolism in rat liver mitochondria. The aim of this study was to evaluate the effect of pharmacological treatment with fluoxetine during critical period for development on the mitochondrial bioenergetics and oxidative stress in liver of rat adult. To perform this study, the rat pups received Fx, or vehicle (Ct) from postnatal day 1 to postnatal day 21 (ie, during lactation period). We evaluated mitochondrial oxygen consumption, respiratory control ratio, ROS production, mitochondrial swelling by pore opening, oxidative stress biomarkers, and antioxidant defense in liver of rats at 60 days of age. Our studies have shown, that treatment with Fx during the lactation period resulted in reduced body mass gain, improvement of the mitochondrial respiratory capacity, induced higher mitochondrial resistance to calcium ion preventing the mitochondrial permeability transition pore opening, as well as decreased oxidative stress biomarkers, and increased the SH levels and enzymes antioxidant activities (SOD, CAT, GST) in liver of treated rats at 60 days of age. These findings suggest that pharmacological treatment with fluoxetine during critical period of development result in positive changes in liver of rats, as improvement of the mitochondrial bioenergetics and hepatic oxidative metabolism that persist in adulthood.

KEYWORDS
critical period of development, fluoxetine, mitochondria, oxidative stress, rat liver, serotonin

1 | INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are a class of antidepressants such as fluoxetine, citalopram, sertraline among others, that are often prescribed to pregnant and lactating women with varying degrees of depression, thus exposing fetuses, and infants to drug during critical periods of development.[1] Some authors demonstrate that fluoxetine (Fx)

Claudia J. Lagranha, Mariana P. Fernandes equally contribute for this manuscript.

Mitochondrial dysfunction: maternal protein restriction as a trigger of reactive species overproduction and brainstem energy failure in male offspring brainstem

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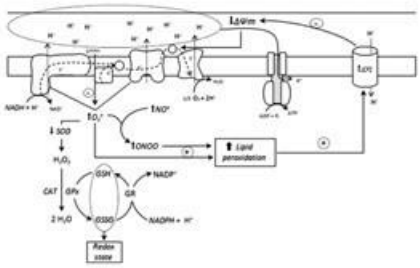
Mitochondria are important organelles in eukaryotic organisms, wherein their capacity to produce energy vary among the tissues depending upon the amounts of oxygen consumed. Part of the oxygen consumed during ATP generation produces reactive oxygen species, which if not efficiently removed can trigger a systemic damage to molecular compounds characterized as oxidative stress. Several studies have demonstrated that mitochondrial dysfunction and oxidative stress in the central nervous system (CNS) are related to a plethora of neural disorders. Herein, we hypothesize that a late autonomic imbalance-induced hypertension might be related to long-lasting effects of protein restriction during the critical period of the CNS development on the mitochondrial function and oxidative stress in the brainstem of adult (i.e. 150 days of age) male *Wistar* rats. Maternal protein restriction was induced by offering a diet based on 8% of casein from first day of pregnancy until weaning, when the male pups started to receive laboratory chow up to 150 days of life. The protein restriction induced an extended detrimental modulation in mitochondria function, decreasing the phosphorylation capacity with concomitant decrease in the mitochondrial membrane potential, wherein the reactive species overproduction triggered a disruption in proton conductance, which may gradually compromise mitochondria energy conservation. Interestingly, the elevated activity of glutathione-S-transferase and the augmented expression of uncoupling protein 2 are likely protective mechanisms induced by lipid peroxidation products, being feasible molecular changes attempting to deal with oxidative stress-induced ageing.



Keywords: Brainstem, Mitochondria bioenergetics, Oxidative stress, Protein restriction, Reactive oxygen/nitrogen species

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Introduction

Mitochondria are dynamic organelles that perform several interconnected actions.[1] In eukaryotic cells, mitochondria represent the most important ATP

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ORIGINAL ARTICLE

Anacardic Acids from Cashew Nuts Prevent Behavioral Changes and Oxidative Stress Induced by Rotenone in a Rat Model of Parkinson's Disease

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Abstract
Anacardic acids (AAs) are alkyl phenols mainly presenting in cashew nuts. The antioxidant effects of these compounds have been an area of interest in recent research, with findings suggesting potential therapeutic use for certain diseases. Nevertheless, none of these studies were performed in order to test the hypothesis of whether anacardic acids are capable of preventing behavioral changes and oxidative stress induced by the pesticide rotenone in experimental model of Parkinson's disease. In our research, adult male rats were treated orally with AAs (1, 3, 10, 25, 50, or 100 mg/kg/day) 1 h before rotenone (3 mg/kg; s.c.) for five consecutive days. The behavioral testing strategies, including tests for general locomotor activity (open field), motor coordination (rotarod), and spatial memory performance (elevated T-maze), were carried out. Lipoperoxidation levels and total superoxide dismutase (t-SOD) activity, as well as cytoplasmic and mitochondrial SOD gene expression, were assessed in the substantia nigra (SN), striatum, and cerebral cortex. The results showed that AAs dose-dependently prevented the rotenone-induced learning and motor impairment from 10 mg/kg/day. AAs also precluded rotenone-induced lipoperoxidation in all doses, acting directly on the mitochondria, and improved the t-SOD activity in the doses 25–100 mg/kg/day. AAs per se (100 mg/kg/day) increased SOD gene expression and t-SOD activity. Our findings indicate that the oral administration of AAs prevents rotenone-induced behavioral changes and oxidative stress, in part due to a modulatory action on the mitochondria and SOD gene expression. These data suggest that AAs have promising neuroprotective action against degenerative changes in Parkinson's disease.

Keywords Rotenone · Lipoperoxidation · Superoxide dismutase · Substantia nigra · Motor behavior

Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative disorder characterized by motor, limbic and cognitive impairment that negatively affects the quality of life during aging (Jankovic 2008; de Farias et al. 2016). Population-based studies have indicated that the number of individuals looking for treatment for PD is likely to increase significantly over the

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Review article

Protective effects of estrogen against cardiovascular disease mediated via oxidative stress in the brain☆

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Keywords:
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ABSTRACT

During their reproductive years women produce significant levels of estrogens, predominantly in the form of estradiol, that are thought to play an important role in cardioprotection. Mechanisms underlying this action include both estrogen-mediated changes in gene expression, and post-transcriptional activation of protein signaling cascades in the heart and in neural centers controlling cardiovascular function, in particular, in the brainstem. There, specific neurons, especially those of the bulbar region play an important role in the neuronal control of the cardiovascular system because they control the outflow of sympathetic activity and parasympathetic activity as well as the reception of chemical and mechanical signals. In the present review, we discuss how estrogens exert their cardioprotective effect in part by modulating the actions of internally generated products of cellular oxidation such as reactive oxygen species (ROS) in brain stem neurons. The significance of this review is in integrating the literature of oxidative damage in the brain with the literature of neuroprotection by estrogen in order to better understand both the benefits and limitations of using this hormone to prevent cardiovascular disease.

1. Introduction

During a 30–40 year reproductive period beginning with menarche and ending with menopause, women produce in a cyclic fashion a number of steroid, protein, and small polypeptide hormones important for the onset and maintenance of reproductive capability, and the development of secondary sexual characteristics. Although classically associated with their role in the female reproductive cycle, estrogens are increasingly being recognized for their protective actions against chronic and degenerative diseases affecting the cardiovascular system [1] and other organ systems that are not directly related to reproduction. This non-reproductive role for estrogens may underlie the long-recognized advantages that women have over men in retaining their general health and in attaining greater longevity.

The term 'estrogen' refers to a small group of steroidal hormones important in the control of the reproductive cycle. The three major estrogens are estrone (E1), 17-β-estradiol (E2) and estriol (E3) which differ from each other in the number of hydroxyl groups or ketones [2] present, but which exhibit to varying degrees the same effects on target reproductive organs and secondary sexual characteristics. 17-β-estradiol is the predominant estrogen during the reproductive years, both in its total serum concentration and in overall estrogenic activity. Most of the circulating estrogen in premenopausal women is produced by the ovaries, largely as a result of secretion from the granulosa cells of developing follicles, and during pregnancy is supplemented by estrogen secretion from the placenta. Estrogens are also produced in small quantities by several peripheral tissues such as liver, adrenal gland, mammary glands, adipose tissue and brain. Similar to ovarian granulosa cells, the expression of the enzyme aromatase in these tissues allows local conversion of C-19 steroids such as antrostenediol to estrogens, although at much lower levels than in the ovary [3,4].

In the perimenopausal period and during menopause, there is a massive reduction in the production of estrogens due to the exhaustion of ovarian follicles and consequently, a failure of the gonadotropins

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