Aline Isabel da Silva

Inibição da Recaptação da Serotonina Durante o Desenvolvimento: Um Estudo do Balanço Energético e da Função Mitocondrial

Recife-2014

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Tese apresentada ao Programa de Pós-Graduação em Nutrição do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, para obtenção do título de Doutor em Nutrição

Orientador : Raul Manhães de Castro

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Aline Isabel da Silva

INIBIÇÃO DA RECAPTAÇÃO DA SEROTONINA DURANTE O DESENVOLVIMENTO: UM ESTUDO DO BALANÇO ENERGÉTICO E DA FUNÇÃO MITOCONDRIAL

Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Nutrição pela Universidade Federal de Pernambuco.

Aprovado por:

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Recife, 2014.

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"...Em todas as circustâncias, dai graças, porque esta é a vontade de Deus a vosso respeito..."(I Tessalonicenses 5:18).

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A Idade de Ser Feliz

“ Existe somente uma idade para a gente ser feliz,somente uma época na vida de cada pessoa em que é possível sonhar e fazer planos e ter energia bastante para realizá-los a despeito de todas as dificuldades e obstáculos. Uma só idade para a gente se encontrar com a vida e viver apaixonadamente e desfrutar tudo com toda intensidade sem medo nem culpa de sentir prazer. Fases douradas em que a gente pode criar e recriar a vida à nossa própria imagem e semelhança e vestir-se com todas as cores e experimentar todos os sabores e entregar-se a todos os amores sem preconceito nem pudor. Tempo de entusiasmo e coragem em que todo desafio é mais um convite à luta que a gente enfrenta com toda disposição de tentar algo novo, de novo e de novo, e quantas vezes for preciso. Essa idade tão fugaz na vida da gente chama-se ***presente*** *e tem a duração do instante que passa.”*

-Mario Quintana

Resumo

Os neurônios serotoninérgicos, presentes no cérebro desde o início do desenvolvimento, estabelece diversos e complexos fenótipos celulares e interações neurais dentro da arquitetura dinâmica do cérebro. É evidente que o sistema serotoninérgico e suas propriedades plásticas sejam cruciais para a capacidade do cérebro de se integrar adequadamente com os órgãos periféricos do corpo bem como, com o ambiente externo. Neste sentido, estudos experimentais mostram que a super estimulação ou depressão dos sistemas de neurotransmissores, durante períodos críticos do desenvolvimento, resulta em alterações de componentes cerebrais e que o desenvolvimento do sistema serotoninérgico poderá ter prejuízos permanentes. No entanto, ainda são escassos os estudos que associem essas alterações do sistema serotoninergico com o desequilíbrio energético e o controle de peso corporal. Neste trabalho avaliamos os efeitos crônicos da utilização de inibidor seletivo de recaptação da serotonina sobre o controle do balanço energético e da bioenergética mitocondrial em tecidos central e periféricos de ratos. Em nosso estudo observamos que o tratamento com fluoxetina (grupo Fx) promoveu uma redução no ganho de peso, associado a um menor percentual (25%) de massa gorda, mas nenhuma diferença na ingestão de alimentos na lactação e pós desmame. Não observamos diferenças entre os grupos nas avaliações de medidas basais da atividade locomotora livre e temperatura corporal. Porém, após estímulo térmico (-15ºC) observamos que o grupo fluoxetina perdeu 30% menos calor quando comparado ao grupo controle. Avaliando a bioenergética mitocondrial no hipotálamo, músculo extensor longo dos dedos e tecido adiposo marrom observamos um aumento do consumo de oxigênio, redução da produção das espécies reativas de oxigênio e nenhuma indução de estresse oxidativo em todos os tecidos estudados. Adicionado à esses resultados, no tecido adiposo marrom do grupo fluoxetina observamos um retorno da respiração mitocondrial aos níveis similares ao grupo controle após adição de GDP (inibidor de proteínas desacopladoras-UCP), como também um aumento de 23% na expressão da proteina UCP-1. De forma geral, nossos resultados sugerem que a exposição precoce à fluoxetina reduz o ganho de peso associado a uma modulação positiva da função mitocondrial, o que possivelmente reduziria o risco para o aparecimento e desenvolvimento de doenças na vida adulta.

Palavras-chaves: Fluoxetina. Respiração celular. Estresse oxidativo, Lactação. Serotonina.

Abstract

The serotonergic neurons present in the brain since the beginning of development, establishes several complex interactions within the dynamic architecture of the brain. The serotoninergic system and its plastic properties are crucial to the ability to integrate central and peripheral organs with the external stimulus. In this sense experimental studies have shown that the over stimulation or depression of neurotransmitter systems during critical period of development, results in changes in brain's components and may induce permanent damage in serotonergic system. However there are still few studies that link these changes to energy imbalance and body weight control. In this study we evaluate the effects of chronic use of selective serotonin reuptake inhibitor in the control of energy balance and mitochondrial bioenergetics in central and peripheral rat tissues. In our study we observed that treatment with fluoxetine (Fx group) induces reduction in weight gain, coupled with a lower percentage (25%) of body fat, but no difference was observed on food intake during lactation and post-weaning. No differences were observed on free locomotor activity and thermogenesis without stimulus. However, after thermal stimulation (-15 °C) was observed that the fluoxetine group lost 30% less energy than Control group. Evaluating the mitochondrial bioenergetics in the hypothalamus, extensor digitorum longus muscle and brown adipose tissue was observed an increase in oxygen consumption, reduction in reactive oxygen species production and no induction of oxidative stress in all tissues. Added to these results in brown adipose tissue from fluoxetine group we observe a return to control levels in mitochondrial oxygen consumption after addition of GDP (inhibitor of UCP) and also we verified that fluoxetine treatment increases protein expression of UCP-1 by 23%. Overall our results suggest that early exposure to fluoxetine reduces weight gain associated with up regulation of mitochondrial bioenergetics, which might reduce the risk for the onset and development of diseases later in life.

Keywords: Fluoxetine. Cellular respiration. Oxidative stress, Lactation. Serotonin.

Lista de abreviaturas e siglas

5-HIAA - 5-Hidroxiindolacético

5-HT - Serotonina

5-HTT - 5-Hidroxitriptofano

ADP - Adenosina Difosfato

AgRP - Peptídeo Relacionado ao Gene Agouti

ATP - Adenosina Trifosfato

BSA - Albumina de Soro Bovino

bw/pc- Peso corporal

^{o}C - Graus Celcius

CART - Transcrito Relacionado à Cocaína e Anfetamina

CAT - Catalase

CCCP- Cianeto de carbonil m-clorofenilhidrazona

CDNB - 1-cloro-2,4 dinitrobenzeno

CEUA- Comissão de Ética no uso de Animais

CNS- Sistema Nervoso Central

Ct- Grupo Controle

DNP-SG - Dinitro Fenil S Glutationa

DOB- dias de nascimento (days of birth)

DTT - Ditiotreitol

EDL - Extensor Longo dos Dedos

EDTA - Ácido Etilenodiamino Tetra-Acético

EGTA - Ácido Tetra Acético Etileno Glicol

EPM - Erro Padrão da Média

EROS- Espécies Reativas de Oxigênio

ETC- Cadeia Transportadora de Elétrons

FADH- Flavina Adenina Dinucleotideo Reduzida

Fx- Grupo Fluoxetina

GDP - Guanosina Difosfato

GSH - Glutationa Reduzida

GST - Glutationa-S-Transferase

H_2DCF-DA - 5-(-6)-clorometil-2',7'-diclorodiidrofluoresceina diacetato

HEPES - Ácido 2-(4-(2-hydroxyethyl)piperazin-1-yl) etanosulfônico

ISRS/ SSRIs - Inibidores Seletivos de Recaptação de Serotonina

KCl - Cloreto de Potássio

KH_2PO_4 - Fosfato Monopotássico

MAO - Monoamina Oxidase

MCR - Receptor de Melanocortina

MDA - Malondialdeído

mg- Miligramas

MOPS - Ácido 3-(morpholino) propano sulfônico

MPTP- Poro de transição de permeabilidade de membrana mitocondrial

NaCl - Cloreto de Sódio

NAD- Nicotinamina Adenina Dinucleotideo

NO - Óxido Nítrico

NPY- Neuropeptídeo Y

O_2 - Oxigênio no estado fundamental

PBS- Tampão fosfato de sódio

PMSF - Fenilmetilsulfonilfluoride

POMC - Pró-ópio-melanocortina

ROS - Espécies Reativas de Oxigênio

SNC - Sistema Nervoso Central

SOD - Superóxido Dismutase

TAB- Tecido adiposo branco

TAM/BAT – Tecido adiposo marrom (brown adipose tissue)

TBARS - Substância Reativa ao Ácido Tiobarbitúrico

TG – Triglicerídeos

TPH - Triptofano hidroxilase

Tris-HCl - Ácido Clorídrico (posição Tris)

UCP - Proteína Desacopladora Mitocondrial

α-MSH - α-Melanócito Estimulante

VLDL- lipoproteínas de muito baixa densidade (very low density lipoprotein)

Sumário

1 APRESENTAÇÃO

O conceito de que estímulos ambientais transmitidos a um organismo em desenvolvimento têm a capacidade de afetar seus perfis de desenvolvimento de curto e longo prazo é intuitivamente atraente e, mais importante, apoiada por um crescente corpo de evidências experimentais e observacionais que põe em questão se alterações no sistema serotoninérgico estariam envolvidos com doenças metabólicas na vida adulta. As doenças metabólicas agrupam as patologias que mais matam no mundo. Dentre elas está a obesidade, considerada um importante fator de risco para uma série de doenças crônicas, como diabetes tipo II e eventos cardiovasculares adversos.

O desenvolvimento do sistema nervoso durante os períodos pré- e pós-natal parece ser particularmente vulnerável ao excesso ou deficiência de mediadores químicos. A serotonina por surgir muito precocemente durante o desenvolvimento e por atuar na morfogênese de estágios iniciais da vida, assume papel determinante na regulação de numerosas funções fisiológicas, dentre elas a regulação da ingestão e dispêndio de energia. Estudos mostram que a utilização de inibidores seletivos de recaptação de serotonina, pode promover alterações morfológicas, funcionais e neuroquímicas em componentes cerebrais e que o desenvolvimento do sistema serotoninérgico poderá ter prejuízos permanentes. A fluoxetina é um fármaco amplamente comercializado para o tratamento de distúrbios neurológicos, tais como a depressão e ansiedade e age na fenda sináptica dos neurônios inibindo de forma seletiva a proteína de recaptação da serotonina e desta forma, aumentando a concentração na fenda sináptica desse neurotransmissor. Apesar de alguns achados, pouco se sabe sobre as consequências do uso crônico dos inibidores seletivos de recaptação de serotonina, durante o desenvolvimento, associando ao metabolismo energético do indivíduo.

As múltiplas funções da serotonina durante toda a vida do cérebro (desenvolvimento e envelhecimento) são bastante intrigantes. Alguns autores relacionam as concentrações de serotonina no sistema nervoso ao estresse oxidativo celular, e mostram resultados bastante divergentes a depender do período de manipulação e do tecido avaliado. O estresse oxidativo têm sido correlacionado à grande número de doenças, indicando que as espécies reativas de oxigênio participam diretamente dos mecanismos fisiopatológicos que determinam a continuidade e as complicações presentes nestes processos. O encéfalo se torna um órgão muito sensível ao estresse oxidativo, devido aos baixos níveis de antioxidantes, altos níveis de

ácidos graxos polinsaturados e da grande necessidade de oxigênio nas reações neurobioquímicas.

Diante do exposto, propomos neste trabalho que o bloqueio da proteína de recaptação da serotonina, em períodos precoces do desenvolvimento, promove adaptações neuroquímicas no sistema nervoso que induzem alterações no balanço oxidativo mesmo após descontinuidade do tratamento. Neste sentido levantamos as seguintes hipóteses: A redução no ganho de peso corporal promovida pela inibição da recaptação da serotonina durante o período perinatal é definida por aumento do dispêndio energético associado ao aumento da função respiratória mitocondrial e; A exposição crônica ao inibidor seletivo de recaptação de serotonina, promove redução nas concentrações de espécies reativas de oxigênio no hipotálamo, tecido adiposo marrom e no músculo extensor longo dos dedos.

Para comprovar nossas hipóteses tivemos como:

- **Objetivo Geral-** Avaliar, em ratos, os efeitos da inibição crônica de recaptação da serotonina durante a lactação sobre os mecanismos de controle do balanço energético e bioenergética mitocondrial.
- **Objetivos Específicos-** Avaliar *in vivo,* aos 40 dias de vida, o peso corporal, consumo alimentar de 24 horas pós natal, níveis de atividade voluntária durante 24 horas; temperatura corporal basal e após estímulo térmico; Avaliar *post mortem,* aos 60 dias de vida, a quantidade de tecido branco retroperitoneal e tecido adiposo marrom interescapular; o consumo de oxigênio mitocondrial, a capacidade da abertura do poro de transição de membrana mitocondrial, a produção de espécies reativas de oxigênio, a peroxidação lipídica, a atividade de enzimas antioxidantes do hipotálamo, tecido adiposo marrom e no músculo extensor longo dos dedos e a expressão proteica de UCP-1 no tecido adiposo marrom.

Esta tese deu origem a três artigos científicos que foram submetidos à publicação. O primeiro intitulado "FLUOXETINE TREATMENT OF RAT NEONATES SIGNIFICANTLY REDUCES OXIDATIVE STRESS IN THE HIPPOCAMPUS AND IN BEHAVIORAL INDICATORS OF ANXIETY LATER IN POSTNATAL LIFE" foi submetido e aceito na revista *Canadian Journal Physiology and Pharmacology* (Qualis B1 em Nutrição). Este artigo teve como objetivo investigar os efeitos do tratamento com fluoxetina em relação a comportamentos alimentar e de ansiedade, juntamente com a análise do balanço oxidativo. O segundo artigo intitulado "EFFECT OF FLUOXETINE TREATMENT ON MITOCHONDRIAL BIOENERGETIC IN CENTRAL AND

PERIFERIC RAT TISSUES" foi submetido à revista *Applied Physiology, Nutrition and Metabolism* (Qualis A2 em Nutrição). Nosso segundo artigo original teve como objetivo investigar os efeitos da exposição crônica ao inibidor seletivo de recaptação da serotonina durante a lactação sobre a bioenergética mitocondrial em tecidos central e periférico envolvidos com o balanço energético de ratos. O terceiro artigo intitulado "NEONATAL MANIPULATION OF THE SEROTONIN ALTERS ENERGY BALANCE: PARTICIPATION OF MITOCHONDRIA AND UCP IN BROWN FAT TISSUE", foi submetido à revista *Biochimica et Biophysica Acta- Bioenergetics* (Qualis A1 em Nutrição). Este artigo teve como objetivo investigar os mecanismos responsáveis pela redução de ganho de peso em ratos tratados com fluoxetina durante o período lactacional.

2 REVISÃO DA LITERATURA

2.1 Serotonina na regulação central e periférica do balanço energético

A serotonina ou 5-hidroxitriptamina ou 5-HT, foi descoberta desde o ano de 1930 quando Erspamer começou a estudar a distribuição de um tipo celular, chamada células enterocromafins, que se coravam com um reagente para indóis. As maiores concentrações foram observadas na mucosa gastrointestinal e em seguida nas plaquetas e no Sistema Nervoso Central (SNC) (Erspamer, 1986). A esta substância, eles chamaram de ***enteraminas***. Pager e colaboradores foram os primeiros a isolar e caracterizar quimicamente essa substância que atuava de forma vasoconstrictora e era liberada pelas plaquetas no sangue no processo de coagulação. Eles a denominaram então de serotonina ou simplesmente 5-HT (Rapport *et al.*, 1948). Contudo só em 1976, Pager demonstrou ser, a serotonina, a mesma substância encontrada por Erspamer em 1930.

Os neurônios serotoninérgicos são encontrados numa ampla variedade de organismos. Nos mamíferos, estão entre os primeiros neurônios que são diferenciados durante o desenvolvimento, e compreendem uma complexa rede neuronal distribuídos no cérebro (Mazer *et al.*, 1997; Lesch e Waider, 2012). Vários dados experimentais indicam que a 5-HT pode atuar como uma via de sinalização encefálica do feto durante períodos críticos de desenvolvimento. Reconhece-se que a 5-HT é sintetizada no início do período embrionário e os seus receptores são expressos precocemente. O encéfalo do feto recebe além da 5-HT endógena àquela proveniente da placenta da mãe, enfatizando ainda mais a importância da 5-HT no desenvolvimento embrionário precoce do cérebro. A contribuição dessas interações materno-placentário-fetal parece ser crítica para a formação de circuitos cerebrais e para as suas funções a longo prazo (Sullivan *et al.*, 2011). Estudos utilizando modelos genéticos em ratos revelam que os níveis excessivos de 5-HT no encéfalo alteram o correto desenvolvimento do córtex somatosensorial (Cases *et al.*, 1996; Persico *et al.*, 2001; Dayer, 2014). Por outro lado, a depleção de 5-HT no cérebro leva a defeitos comportamentais e funcionais no SNC (Hendricks *et al.*, 2003; Savelieva *et al.*, 2008; Alenina *et al.*, 2009). Estes dados sugerem que as alterações dos níveis de 5-HT durante o desenvolvimento do SNC produzem alterações permanentes em circuitos serotonérgicos afetando as corretas atividades cerebrais.

Apenas 2% da 5-HT é produzida no SNC, nos núcleos da rafe, localizados no tronco encefálico (Nasyrova *et al.*, 2009). Nos seres humanos, assim como na maioria das outras espécies de mamíferos, a 5-HT pode ser sintetizada a partir do aminoácido essencial triptofano. A primeira etapa de sua síntese pode ocorrer por ação de duas enzimas distintas, a triptofanohidroxilase (TPH) 1 e 2 (Cote *et al.*, 2007). A TPH1 está localizada na glândula pineal e células enterocromafins do intestino sendo responsável por sintetizar a maior parte da serotonina encontrada no organismo. A TPH2, que é restrita aos neurônios dos núcleos da rafe e do sistema nervoso entérico, é responsável pela síntese do restante da serotonina (Erspamer, 1954; Hoyer *et al.*, 2002). Na primeira etapa, o aminoácido essencial é hidroxilado pela enzima TPH tendo como produto o 5-hidroxitriptofano (5-HTT). Na sequência, o 5-hidroxitriptofano é descarboxilado pela triptofano descarboxilase, formando a 5-HT (Clark *et al.*, 1954).

No cérebro de mamíferos os neurônios serotoninérgicos exercem os seus efeitos através de 20 subtipos de receptores que são agrupados em sete classes distintas (5-HT_1 a 5-HT_7) com base nas propriedades farmacológicas, nas sequências de aminoácidos, organização de genes e nas vias acopladas de segundo mensageiro (Hoyer *et al.*, 1994; Gellynck *et al.*, 2013; Volpicelli *et al.*, 2014). Os tipos e subtipos de receptores da 5-HT estão acoplados a diferentes mecanismos de sinalização transmembrana e diversos são os fatores que determinam a intensidade e duração da sinalização desses receptores, sendo a quantidade de 5-HT liberada na fenda sináptica o principal deles (Cerrito e Raiteri, 1979).

A ação da 5-HT pode ser finalizada por sua recaptura da fenda sináptica ao botão pré-sináptico, através de proteínas transportadoras localizadas na membrana de neurônios pré-sinápticos. Essas proteínas transportadoras são alvo de alguns fármacos utilizados para aumentar a concentração de 5-HT para o processo de neurotransmissão (Wong e Bymaster, 1995). Uma vez no espaço intracelular a 5-HT pode ser metabolizada a partir da ação da monoamina oxidase (MAO), uma flavoenzima localizada na membrana das mitocôndrias (Sandler *et al.*, 1981). Existem dois tipos de MAO, a MAO-A e B. A primeira é responsável pela metabolização da 5-HT encefálica. A MAO-B age primordialmente sobre a 5-HT periférica (plaquetas, células enterocromafins). A 5-HT sofre ação da MAO formando o aldeído 5-hidroxindolacetaldeído que por sua vez pode ser convertido em ácido 5-hidroxiindolacético (5-HIAA) pela enzima aldeído desidrogenase ou por uma via alternativa que consiste na sua redução pela ação da enzima aldeído redutase do acetaldeído a álcool, o 5-hidroxitriptofol. No entanto, esta via é normalmente insignificante. O 5-HIAA do cérebro e dos locais periféricos de armazenamento e metabolismo da 5-HT é excretado na urina

juntamente com pequenas quantidades de sulfato de 5-hidroxitriptofol ou conjugados de glicuronídeos (Sandler *et al.*, 1981).

Dentre as funções da 5-HT está a regulação do balanço energético (Heisler *et al.*, 2006; Vickers *et al.*, 2008). A manutenção do balanço energético requer diversos ajustes fisiológicos e comportamentais visando à obtenção de energia, sua metabolização e seu armazenamento (Williams *et al.*, 2012; Jeong *et al.*, 2014). Diversos sinais do estado nutricional e do nível de energia do organismo são integrados no SNC para produção de respostas adequadas para manutenção da homeostase energética (Guyenet e Schwartz, 2012). Em humanos, o estado das reservas energéticas é sinalizado ao cérebro pelos sinais da adiposidade e saciedade. Estes sinais modificam tanto as vias anabólicas como as catabólicas, alterando o comportamento de ingestão alimentar e o tamanho da refeição de acordo com o requerimento energético sinalizado (Grill, 2010; Abizaid e Horvath, 2012; Keen-Rhinehart *et al.*, 2013).

A energia que nosso corpo dissipa (gasto de energia) é representada pela soma de calor interno produzido e do trabalho externo. O calor interno produzido, por sua vez, é a soma da taxa metabólica basal e o efeito térmico dos alimentos e o trabalho externo, podendo ser estimado pelo nível de atividade física (Wynne *et al.*, 2005). Uma população de neurônios, localizado no núcleo arqueado, que expressa o neuropeptídeo proopiomelanocortina (POMC) parece ter um papel especialmente importante na mediação de sinais do balanço energetico por vias serotoninérgicas. O núcleo arqueado está localizado numa região altamente vascularizada do hipotálamo, imediatamente adjacente à eminência mediana, permitindo o acesso a vários fatores circulantes (Cone *et al.*, 2001; Rodriguez *et al.*, 2010). O hipotálamo é constituído por vários núcleos, que corresponde a conjuntos de corpos de neurônios dispersos em uma rede de substância branca (conjunto de axônios). Esta região do SNC compreende um centro integrador dos sinais de saciedade ou de fome provenientes do trato gastrointestinal, do pâncreas, do fígado e ou do tecido adiposo (Morton *et al.*, 2006). Desta forma, os neurônios POMC, dentro do núcleo arqueado, desempenham um papel importante na integração de estímulos periféricos (Williams e Elmquist, 2011). A 5-HT é capaz de ativar esse conjunto celular, através de receptores serotoninérgicos específicos, o 5-HT_{1B} e $_{2C}$, aumentando a expressão do neuropeptídeo POMC nos neurônios (Simansky, 1996; Halford and Blundell, 1996; Heisler *et al.*, 2002; Berglund *et al.*, 2013).

As populações de neurônios hipotalâmicos que sintetizam POMC emitem projeções para neurônios pré-ganglionares, localizados na coluna mediolateral da medula espinal, e estes se comunicam com o músculo esquelético por meio de fibras pós-ganglionares

simpáticas (Broberger, 2005). Há evidências de quando esta via é estimulada ocorrem mudanças no metabolismo energético do músculo esquelético resultando em maior disponibilidade de ácidos graxos para serem oxidados através da β– oxidação mitocondrial (Cha *et al.*, 2005; Cha *et al.*, 2006). Mais recentemente, estudos mostram que o hipotálamo influencia no metabolismo dos triglicerídeos (TG) através de sua inervação para o fígado, tedido adiposo marrom (TAM) e tecido adiposo branco (TAB), principalmente por vias simpáticas do sistema nervoso autônomo (Brito *et al.*, 2007; Bruinstroop *et al.*, 2012; Geerling *et al.*, 2014).

A dependência que os mecanismos de controle do balanço energético têm do sistema nervoso central, demonstra as vulnerabilidades do organismo durante o período crítico do desenvolvimento às informações ambientais e sua capacidade de adaptação a estímulos diversos (Bellinger *et al.*, 2004).

2.2 Mitocôndria, UCPs, Estresse Oxidativo e Serotonina

A mitocôndria é uma organela celular importante, sendo responsável por muitos processos fundamentais para a obtenção de energia para a célula, como a β-oxidação de ácidos graxos, o Ciclo de Krebs e a Cadeia respiratória (Brand e Nicholls, 2011). Todas as células contam com um fornecimento externo de energia oriunda dos alimentos, como carboidratos e lipídios. Esses combustíveis são transportados para as células e passam por uma série de reações metabólicas. Na maioria dos casos, intermediários metabólicos das vias citosólicas são transportados para as mitocôndrias onde ocorre a fosforilação oxidativa. A energia que é liberada nesta via é armazenada na forma de Adenosina Trifosfato (ATP), um intermediário rico em energia que é utilizado como a moeda energética em um grande repertório de reações (Hepple, 2014).

Nem toda a energia é armazenada como ATP, por exemplo no tecido adiposo marrom, a energia que é derivada de combustíveis metabólicos é dissipada em um processo que é facilitado por um escape de prótons, liberando assim o calor. A regulação do escape de prótons neste tecido é mediada pelas proteínas desacopladoras mitocondriais 1 (UCP1) que estão localizadas na membrana mitocondrial interna dos adipócitos marrons (Krauss *et al.*, 2005). Desta forma, as proteínas desacopladoras mitocondriais (UCPs) desempenham um papel proeminente na regulação da termogênese e balanço energético (Adams, 2000; Silva *et al.*, 2005). A UCP1 é um membro da família do gene das UCPs, que contém dois homólogos

estreitamente relacionadas, a UCP2 e UCP3 (55% e 56% de homologia respectivamente) (Hughes e Criscuolo, 2008). A UCP-1 é o único gene conhecido por ser expresso exclusivamente no TAM sendo responsável por até 5% do total de proteína mitocondrial neste tecido (Brand *et al.*, 1999). A UCP-2 é expressa em vários tecidos (sistema nervoso central, pâncreas, coração, fígado, entre outros) e a UCP-3 é principalmente expressa no músculo esquelético e no tecido adiposo marrom de humanos e roedores (Clapham *et al.*, 2000; Toda e Diano, 2014). O sistema serotoninérgico parece ter relação direta com a expressão de genes mitocondriais responsáveis pela produção das proteínas desacopladoras mitocondriais (UCPs). Em um estudo de Nonogaki *et al*. (2002) camundongos com mutação nos receptores $5HT_{2C}$ apresentaram redução no consumo de oxigênio e aumento nos níveis de RNAm UCP-2 no fígado, tecido adiposo branco e músculo esquelético e redução na expressão do receptor β3-adrenérgico. Esses resultados sugerem que alteração no receptor de 5-HT induz aumento na adiposidade por modulação de vários genes (Nonogaki *et al.*, 2002).

Alguns estudos evidenciam que as UCPs podem atuar modulando a produção de espécies reativas de oxigênio (EROS) na mitocôndria (Brand *et al.*, 2002). A mitocôndria é uma das principais fontes geradoras de EROS. Define-se como EROS toda espécie de oxigênio que possui um ou mais elétrons desemparelhados. O elétron livre, que caracteriza a espécie reativa, pode estar centrado em um átomo de hidrogênio, oxigênio, nitrogênio, carbono, enxofre ou átomos de metais de transição. Pelo fato da molécula de oxigênio ser um bi-radical (possuir dois elétrons livres nos orbitais p antiligantes), o oxigênio reage preferencialmente com moléculas de configuração eletrônica semelhante. Como a maioria das biomoléculas não são bi-radicais, possuindo grande número de ligações covalentes, o oxigênio fica impedido (por restrição de "spin") de reagir com as mesmas, evitando assim que alvos celulares importantes sejam lesados (Sies e Mehlhorn, 1986; Halliwell e Aruoma, 1991).

Uma via de formação de espécies reativas de oxigênio consiste na redução monoeletrônica do oxigênio à água, na qual a entrada sequencial de elétrons na molécula de oxigênio promove a formação do radical superóxido (O_2^-) e que pela ação das enzimas antioxidantes ou reações com metais forma o peróxido de hidrogênio (H_2O_2) e o radical hidroxila (OH^-) (Sies, 1991). O radical hidroxila é, entre as ROS conhecidas, uma das mais reativas, pois necessita somente de mais um elétron para se estabilizar. Estas ROS para se estabilizarem devem doar ou receber elétrons de uma ou mais moléculas, levando a oxidação de fosfolipídios de membranas celulares e subcelulares, ou de proteínas e ou mesmo do DNA (Halliwell e Aruoma, 1991). Portanto, a toxicidade do oxigênio, decorre da formação de EROS que podem

interagir com diversas biomoléculas, com o objetivo de se estabilizarem lesando diferentes estruturas celulares (Greenwald, 1990).

Estudos evidenciaram que a patogênese de diversas doenças neurodegenerativas, incluindo a doença de Parkinson, doença de Alzheimer, ataxia de Friedreich, esclerose múltipla e esclerose lateral amiotrófica, pode envolver a geração de EROS e/ou espécies reativas de nitrogênio associada à disfunção mitocondrial (Gutteridge, 1993). Sob condições fisiológicas normais as EROS exercem papel importante atuando nos mecanismos de reações inflamatórias ou também como segundos mensageiros intracelulares mantendo diversas funções celulares (Rosen *et al.*, 1995; Blake *et al.*, 1994). Assim, o equilíbrio entre a formação e a remoção das EROS no organismo deve ser altamente regulado, de forma que as reações e processos metabólicos dependentes das mesmas possam ocorrer adequadamente para a manutenção dos processos fisiológicos celulares (Blake *et al.*, 1994).

O desequilíbrio entre a formação e a remoção das EROS no organismo, decorrente da diminuição dos antioxidantes endógenos ou do aumento da geração de espécies oxidantes, gera um estado pró-oxidante que favorece a ocorrência de lesões oxidativas em macromoléculas e estruturas celulares, inclusive podendo resultar na morte celular (Gutteridge, 1993). Este tipo de lesão oxidativa é definida como estresse oxidativo, uma condição na qual ocorre um desequilíbrio entre as concentrações de espécies pró-oxidantes em detrimentos das concentrações antioxidantes (Sies e Mehlhorn, 1986).

Neste contexto, os dois principais meios de defesa antioxidantes no organismo podem ser divididos em dois grupos, enzimáticos e não enzimáticos. Os sistemas enzimáticos envolvem várias enzimas, tais como as enzimas do ciclo das glutationas, particularmente a glutationa peroxidase e glutationa S-transferase. Outros sistemas enzimáticos de defesa antioxidantes operando em conjunto com as enzimas citadas anteriormente incluem a superóxido dismutase (SOD), dependente de Cu^{2+} e Zn^{2+} como cofatores, onde cataliza a dismutação do radical superóxido em peróxido de hidrogênio (H_2O_2) e oxigênio, bem como a catalase, que converte peróxido de hidrogênio em água e oxigênio molecular (Meister e Anderson, 1983).

Na literatura já existem relatos da relação entre concentrações de serotonina e estresse oxidativo, no entanto, esses mostram resultados bastante divergentes. Os modelos experimentais que alteram as concentrações de serotonina, utilizando Inibidores Seletivos de Recaptação (ISRS), mostraram que a ação deste fármaco foi capaz de reduzir os níveis de EROS, agindo pois, como agente antioxidante (Khanzode *et al.*, 2003; Zafir *et al.*, 2009; Ahmad *et al.*, 2010; Moretti *et al.*, 2012). Em outro estudo, os autores mostram que ratos adultos expostos a fluoxetina apresentam uma produção elevada de EROS e que o sistema de

defesa antioxidante no fígado está afetado. Os autores sugerem que isso possivelmente ocorra porque o fígado é o local de metabolização da fluoxetina, o que aumentaria os níveis de metabólitos ativos da 5-HT e lesão tecidual (Inkielewicz-Stepniak, 2011). Além destes trabalhos, foi demonstrado que a utilização crônica de fluoxetina promoveu aumento nos indicadores de apoptose e na fragmentação do DNA também no fígado (Djordjevic *et al.*, 2011). Em outro estudo trabalhando com os antidepressivos clomipramina, norfloxetina e desipramina, mas em cultura de células cardíacas, os pesquisadores demonstraram que os antidepressivos promoveram aumento da morte celular por apoptose, e atribuem esse dano ao rompimento da função mitocondrial resultante da inibição de vários complexos enzimáticos (Abdel-Razaq *et al.*, 2011). Por fim, em um estudo com o baço, os autores sugerem que a fluoxetina após um tratamento de 14 dias não altera os sistemas de defesa nem a peroxidação lipídica neste órgão (Kirkova *et al.*, 2010).

A partir das evidências existentes observamos que os dados a respeito do papel da serotonina em relação ao estresse oxidativo celular ainda não foi totalmente elucidado. Em adição, até onde verificamos nenhum estudo com animais de experimentação foi realizado com o intuito de investigar o papel da serotonina durante o desenvolvimento sobre a função mitocondrial no que concerne á produção de espécies reativas de oxigênio em tecidos chaves no controle do metabolismo energético como hipotálamo, tecido adiposo marrom e músculo esquelético.

Diante do exposto nessa revisão, é necessário um maior entendimento a cerca dos mecanismos de controle do balanço energético a fim de gerar mais subsídios para futuras ações intervencionistas que resultem na redução do número de indivíduos acometidos por doenças metabólicas.

3 MATERIAIS E MÉTODOS

3.1 Animais

Foram utilizados ratos da linhagem *Wistar*, sendo oito fêmeas e oito machos provenientes da colônia do Departamento de Nutrição da Universidade Federal de Pernambuco. Os filhotes machos (n=64) oriundos do acasalamento entre os animais adultos foram utilizados no presente estudo.

Os animais foram obtidos através de critérios (idade, peso e grau de parentesco) pré-estabelecidos, o que implicou em um trabalho sob ambiente controlado e padronizado. Contudo, para evitar contaminações e interferências nos experimentos, além de ter o bem estar do animal como prioridade, foi estabelecido um padrão sanitário definido (Faria, 1998; Chorilli *et al.*, 2007).

As fêmeas, para acasalamento (peso corporal de 220-250g) foram abrigadas em biotério sob condições padrão de temperatura, iluminação e umidade com água e comida (dieta Labina – Purina S/A durante todo experimento) *ad libitum*. Em se tratando do controle da temperatura, sabe-se que a sinergia com a umidade do ambiente promove o equilíbrio térmico do animal. As mudanças nesses padrões levam a respostas adaptativas com alterações comportamentais, fisiológicas e metabólicas. A maioria dos animais de laboratório apresenta sudoração insignificante e usa taquipnéia como mecanismo de adaptação frente ao calor. A temperatura e a umidade foram seguidas segundo a recomendação para os ratos de laboratório sendo de 20-24°C e 60% +/- 10%, respectivamente (Van Zutphen, 1993). O monitoramento diário foi estabelecido a fim de evitar estresse térmico. Os animais estão constantemente perdendo calor, umidade e eliminando CO_2, além de outras substâncias resultantes de reações metabólicas, por isso os animais foram mantidos em ambiente onde existe renovação do ar, evitando o acúmulo de substâncias tóxicas nas salas, como a amônia por exemplo. A intensidade da luz e o fotoperíodo influenciam o metabolismo e o ciclo estral dos ratos, alterando suas respostas biológicas (Semple-Rowland e Dawson, 1987). Logo, foram promovidos períodos alternados e regulares de luz e escuridão (12/12 horas) e um período de adaptação de quinze dias na chegada dos animais ao biotério de experimentação, permitindo uma sincronização do ritmo circadiano desses animais.

Após a adaptação, as ratas quando em período estral, foram acasaladas na proporção de uma fêmea para um macho. Para isso foi acompanhado a tipagem das células do epitélio

vaginal por método de esfregaço vaginal em lâmina e posterior observação ao microscópio óptico Leica DMLS. A possível prenhez foi sugerida pela identificação de espermatozóides no esfregaço vaginal (Marcondes *et al.*, 2002). Confirmado o acasalamento, foram consideradas prenhas as ratas que apresentaram aumento diário no peso corporal. As ratas foram mantidas em gaiolas individuais (policarbonato cristal, 49 x 34 x 32) e em condições padrão de biotério durante todo período de gestação.

Os filhotes foram escolhidos de modo aleatório um dia após o nascimento com peso entre seis e oito gramas (Bento-Santos *et al.*, 2012). A ninhada foi formada por oito neonatos os quais foram mantidos com as suas nutrizes até o 21º dia de vida pós natal. Os filhotes restantes da ninhada foram eutanasiados. Após o desmame, os filhotes foram alocados em gaiolas individuais nas mesmas condições padrões de biotério até o final do período experimental. A gaiola era para rato individual produzida em policarbonato cristal transparente, autoclavável e resistente a ácidos, nas medidas de 30x20x19. A cama dos animais foi composta de maravalha de madeira de pinho autoclavada. Após processo de secagem a maravalha foi devidamente peneirada para retirada do pó. O manejo e os cuidados, que se seguiu, foram aprovados pela Comissão de Ética no uso de Animais (CEUA) da Universidade Federal de Pernambuco no processo nº 23076.015276/2012-56 (ANEXO 1).

3.2 Tratamento

3.2.1 ***Farmacológico***

Para manipular o sistema serotoninérgico, foi utilizado durante o período de lactação (1º ao 21º dia de vida) ISRS, a fluoxetina (Farmácia de Manipulação-Roval). Este fármaco bloqueia a proteína transportadora da 5-HT, da fenda ao botão neuronal pré-sináptico, aumentando sua disponibilidade para o processo de neurotransmissão (Hiemke e Hartter, 2000; Qu *et al.*, 2009). A fluoxetina foi escolhido devido suas seletividade e propriedades farmacocinéticas, pois possui ampla absorção e um tempo de ½ vida longo (aproximadamente 4 horas). Além disso seu metabólito, a norfloxetina, também atua inibindo a recaptação de 5-HT e possui um tempo de meia vida mais prolongado que a fluoxetina (aproximadamente 13 horas) e não promove efeitos secundários (Wong e Bymaster, 1995; Qu *et al.*, 2009). Foi utilizado na concentração de 10 mg/Kg de peso corporal (p.c.), a qual já foi observada aumentar as concentrações encefálicas da 5-HT em 1 (uma) hora após administração (Miller

et al., 2008). A droga foi obtida na forma de cloridrato de fluoxetina e dissolvida em veículo controle (1:1), uma solução salina (NaCl) a 0,9%.

3.2.2 Controle

Foi utilizado 10ml/kg p.c. de solução de Cloreto de Sódio (NaCl) a 0,9%.

3.3 Via de Manipulação

O tratamento foi administrado por via subcutânea (sc) e o horário de aplicação dos animais correspondeu a segunda hora após início do ciclo escuro. O horário de manipulação farmacológica foi mantido durante todo o experimento em concordância com o horário do segundo e maior pico de liberação da serotonina (Sanchez *et al.*, 2008). Esse método consistiu na injeção da solução sob a pele do animal, a qual foi levantada antes da aplicação. Foi realizado com agulha hipodérmica curta (normalmente 25 x 5 mm ou mais fina), passando apenas pela derme, o mais próximo da superfície, formando uma pápula após a administração da substância. A área dorsal foi a região de escolha. Essa via raramente induz dor e foi realizada com o animal consciente. Antes de injetar a solução, foi aspirado sob leve pressão o êmbolo da seringa para assegurar que a agulha não esteve penetrando em um vaso sangüíneo.

3.4 Grupos experimentais

No período de lactação foram formados dois grupos experimentais segundo o tratamento:

- **Grupo Controle** (C, n=32): os animais foram tratados diariamente com solução salina a 0,9%, 10ml/kg pc, sc, do 1º ao 21º dia pós-natal;
- **Grupo Fluoxetina** (Fx, n=32): os animais foram tratados com fluoxetina na dose de 10mg/kg pc, sc.; do 1º ao 21º dia pós-natal;

Cada ninhada foi formada por 8 (oito) animais sendo 4 animais do grupo fluoxetina (Fx) e 4 animais do grupo salina (C).

3.5 Procedimentos

3.5.1 Medidas de peso corporal

O peso corporal dos filhotes foi mensurado diariamente (g) durante o período de lactação e também no 40º e 60º dia de vida. O peso foi registrado no início do ciclo claro/escuro através de balança eletrônica digital (Marte, modelo S-100 com sensibilidade de 0.01g) (Da Silva *et al.*, 2014).

3.5.2 Avaliação do consumo alimentar de 24 horas

No 40º dia de vida, os animais dos grupos experimentais C e Fx tiveram seu consumo alimentar de 24h avaliado. Os animais foram separados após desmame (21º dia) e foram submetidos à dieta padrão de biotério *ad libitum* durante todo período experimetal. A ingestão alimentar (g) foi acompanhada a cada 24 h durante 5 dias. Foi oferecida uma quantidade conhecida de ração (R1) e após 24 horas a ração foi novamente pesada (R2). A ingestão alimentar foi dada pela diferença entre R1 e R2 (R1 – R2) sendo utilizado para análise o 5º dia de avaliação (Halford *et al.*, 1998).

3.5.3 Avaliação da temperatura retal

No 40º dia de vida animais dos grupos experimentais C e Fx tiveram sua temperatura corporal avaliada. A mensuração da temperatura corporal dos ratos foi realizada pela via retal. Os animais tiveram a cauda elevada e 1,5 cm da ponta do termômetro clínico digital, lubrificados com óleo mineral, foi mantido no canal retal do animal por 1 minuto. Após registro da temperatura inicial os animais foram alocados em recipiente com ventilação de ar e em seguida submetidos a uma câmara refrigerada com temperatura de -15ºC. A temperatura foi controlada com auxílio de um termômetro digital (Incoterm). As medidas da temperatura retal foram novamente mensurada após tempos progressivos de 30 minutos atingindo um tempo total de 90 minutos (Zheng *et al.*, 2008).

3.5.4 Avaliação da atividade voluntária por 24 horas

No 40º dia de vida, os animais tiveram sua atividade voluntária avaliada por 24 horas. Para esta avaliação os animais precisaram estar alocados individualmente em gaiola de acrílico em pelo menos cinco dias antes da análise. Os animais foram filmados, durante 24 horas, por câmera de infra-vermelho (1/3 480 linha chipsony) e as imagens captadas foram armazenadas em sistema computacional. Posteriormente foi registrado, em segundos, os comportamentos que exprimem atividade voluntária como exploração da gaiola, construção do ninho e comportamento de limpeza (Halford *et al.*, 1998).

3.5.5 Coleta do material biológico

Aos 60 dias de vida, os animais foram anestesiados com Xilasina e Ketamina (ketamina/xilasina, 60:5 mg/kg peso corporal, i.p) para dissecar do encéfalo o hipotálamo, da região retroperitoneal a gordura branca, da região interescapular a gordura marrom e das patas posteriores os músculos esquelético extensor longo dos dedos. Os tecidos retirados do animal foram utilizados para análises bioquímicas, identificação e quantificação de proteína e avaliação da função mitocondrial, estando as metodologias de análise descritas a seguir (Da Silva *et al.*, 2014).

3.5.6 Quantificação do tecido adiposo

A quantificação do tecido adiposo branco e marrom foi realizada através do peso úmido (g) registrado imediatamente após retirada da cavidade retroperitoneal e interescapular respectivamente, com auxílio de balança digital (Marte, modelo S-100 com sensibilidade de 0.01g). (Bugge A, Dib L, Collins S., 2014)

3.5.7 Processamento do material biológico para análise bioquímica

O hipotálamo, o tecido adiposo e muscular coletados foram homogeneizados em tampão de extração (Tris base 100 mM, pH 7,5; EDTA 10 mM; fluoreto de sódio 100 mM;

ortovanadato de sódio 1 mM; PMSF 2 mM). Após a homogeneização, as amostras foram centrifugadas a 4000 rpm, a 4° C, por 10 minutos e o sobrenadante submetido à quantificação de proteína (Da Silva *et al.*, 2014).

3.5.8 Dosagem de proteína

A concentração de proteína da suspensão de cada tecido foi determinada pelo método de Bradford (Bradford, 1976). Este complexo absorve em comprimento de onda de 595nm. A absorbância foi considerada diretamente proporcional à concentração de proteína na solução analisada, onde uma solução de albumina de soro bovino (BSA) foi utilizada como padrão.

3.5.9 Medida dos níveis de estresse oxidativo pela metodologia da Substância Reativa ao Ácido Tiobarbitúrico (TBARS)

Para a dosagem de TBARS foi utilizada a técnica colorimétrica de Buege & Aust (Buege e Aust, 1978), uma técnica muito utilizada para avaliar a lipoperoxidação, pois o ácido tiobarbitúrico reage com os produtos da lipoperoxidação, entre eles o malondialdeído (MDA) e outros aldeídos. Colocou-se uma alíquota do homogenizado, de ácido tricloroacético a 30% e de ácido tiobarbiturico a 0,73% para reagir com os produtos da lipoperoxidação e assim formar um composto de coloração rosada. A mistura foi incubada por 15 minutos a 100ºC e em seguida resfriada. Na sequência, foi adicionado n-butanol (100%) na prorporção de 1:1, e as amostras agitadas por 30 segundos, com o objetivo de extrair o pigmento formado. O material foi centrifugado a 3000 rpm por 10 minutos, sendo então a fase com o n-butanol utilizada para a leitura da absorbância a 535nm, utilizando cubetas de quartzo. A peroxidação lipídica foi avaliada através da quantidade de MDA formado, estando os resultados expressos em nmol/mg proteína (Da Silva *et al.*, 2014).

3.5.10 Atividade enzimatica: Superóxido dismutase (SOD)

A atividade da SOD foi avaliada através do método de auto-oxidação da adrenalina, de acordo com Misra et al. (Misra e Fridovich, 1972). Em uma cuteba de quartzo de 1mL,

adicionou-se tampão carbonato (0.05M, pH=10,2), amostra e adrenalina (3 mM), e incubada por 45 min a 37°C. A absorbância foi registrada por um período de aproximadamente 3 minutos e a atividade da SOD foi determinada pela medida da cinética da inibição da auto-oxidação da adrenalina a 480 nm. Os resultados foram expressos em U/mg proteína (Da Silva *et al.*, 2014).

3.5.11 Atividade enzimática: Catalase (CAT)

A atividade da CAT é diretamente proporcional a taxa de decomposição do peróxido de hidrogênio, sendo assim, a atividade da enzima pode ser medida através da avaliação do consumo de peróxido pelo decréscimo na absorção a 240 nm ([] máx do H_2O_2) de um meio de reação, contendo tampão fosfato (50 mM, pH=7,4) e H_2O_2 (10mM), na temperatura ambiente A atividade da catalase foi expressa em U/mg proteína (Aebi, 1984; Da Silva *et al.*, 2014).

3.5.12 Atividade enzimática: Glutationa-S-Transferase (GST)

A atividade da GST é diretamente proporcional a taxa de formação do composto DNP-SG (dinitro fenil S glutationa), podendo desta forma ser medida através do monitoramento da taxa de formação do composto. Em uma cubeta de quartzo de 1 mL, adicionou-se 800 µL de tampão fosfato (0.2 M, pH 6,5), 100 µL de amostra, 50 µL de GSH (concentração final 1 mM), 50 µL de CDNB (concentração final de 1 mM). A absorbância foi registrada por um período de aproximadamente 3 minutos com controle da temperatura (30^{o}C). Com base na absorbância molecular, 1 unidade de GST é definido como a quantidade de proteína necessária para catalisar a formação de 1 µmol DNP-SG, sendo dessa forma expressa em U/mg de proteína (Habig e Jakoby, 1981).

3.5.13 Isolamento de Mitocôndria

Para isolamento das mitocôndrias os tecidos (hipotálamo, tecido adiposo marrom e músculo EDL) foram homogeneizados, isoladamente, em tampão contendo Manitol (225 mM), Sacarose (75 mM), MOPS (5mM), EGTA (0,5 mM) e 0,02% BSA em solução Tris

Base (pH=7,3) (Lagranha *et al.*, 2010). Foram centrifugados posteriormente a 4.000 rpm por 10 min. O subsequente sobrenadante foi centrifugado a 12.000 rpm por 10 min para a coleta do pellet mitocondrial. O pellet mitocondrial foi resuspendido em 250 mM de sacarose e HEPES a 10 mM a uma concentração final de proteína de 80-100 mg / ml (Leite *et al.*, 2010).

3.5.14 Medida do consumo de oxigênio mitocondrial

O consumo de oxigênio por mitocôndrias foi medido polarograficamente utilizando-se um eletrodo do tipo Clark conectado a um oxímetro, em uma câmara de vidro fechada e termostatizada (1mL), equipada com agitador magnético. Esse tipo de eletrodo compreende um cátodo de platina e um ânodo de prata, imersos numa solução eletrolítica (KCl). A superfície do cátodo é revestida por uma fina membrana de teflon ou polietileno, que são permeáveis ao oxigênio. Quando uma pequena voltagem é aplicada entre os eletrodos, a platina torna-se negativa em relação à prata, tornando-se polarizada. O oxigênio é então reduzido a peróxido de hidrogênio na superfície da platina, funcionando como aceptor de elétrons. A corrente gerada pela diferença dos eletrodos é relacionada estequiometricamente à concentração de O_2 na superfície do cátodo. Os impulsos elétricos são transmitidos ao oxígrafo, onde foi feita a leitura. As mitocôndrias (0,5 mg/ml) foram incubadas em tampão de respiração (pH= 7,24) KCl 120 mmol/L, MOPS 5 mmol/L, EGTA 1 mmol/L, KH_2PO_4 5 mmol/L, e BSA 0,2%. Após a adição de glutamato/malato (10/2 mmol / L) ou succinato (5mM), o estado 3 da respiração foi medido através da adição de ADP (0,5 mmol / L). O estado 4 da respiração mitocondrial foi determinada com a adição de Oligomicina (1,2 μmol / L) (Imahashi *et al.*, 2004; Lagranha *et al.*, 2010). Para as mitocôndrias de tecido adiposo marrom foi adicionado guanidina di-fosfato (GDP, 1mM), um inibidor de UCPs, para avaliar a participação dessas no consumo de oxigênio mitocondrial (Dlaskova *et al.*, 2010).

3.5.15 Estimativa da produção de espécies reativas de oxigênio (EROS)

A produção de EROS em mitocôndrias isoladas foi realizada a 28°C usando probe fluorescente H_2DCF-DA (5- (e 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) o qual é oxidado em presença de peróxido de hidrogênio (Garcia-Ruiz *et al.*, 1995). Rapidamente, a suspensão mitocondrial (0,5 mg protein) foi incubada em presença de

1 µM H_2DCF-DA e a fluorescência foi monitorada durante 5 minutos em baixa agitação usando uma temperatura controlada em espectrofluorímetro (OMEGA, USA) com excitação e emissão de 503 e 529 nm, respectivamente. Sobre essas condições o aumento linear de fluorescência para cada reação foi indicado como a taxa de formação de EROS. A formação de EROS foi medida em mitocôndrias em estado de normoxia com 10/2mM de glutamato/malato.

3.5.16 Avaliação da abertura do poro de transição de permeabilidade de membrana mitocondrial

A abertura do poros de transição de permeabilidade de membrana mitocondrial foi determinada pelo inchamento induzido pelo cálcio em mitocôndrias isoladas de hipotálamo e músculo EDL (Maloyan *et al.*, 2005). A abertura do poro causa inchamento mitocondrial o qual pode ser medido espectrofotometricamente pela redução da absorbância a 540 nm. Mitocôndrias isoladas foram ressuspendidas em tampão de inchamento que contém (em mmol/l) 120 KCl, 10 Tris-HCl (pH 7.4), 20 MOPS, e 5 KH_2PO_4 com concentração final de proteína mitocondrial de 0,25 mg/ml. A abertura do poro foi induzida por cloreto de cálcio a 100µM (Blattner *et al.*, 2001).

3.5.17 Western Blotting para UCP-1

Para análise de expressão proteica, as amostras de mitocôndria foram submetidas à quantificação proteica pelo método de Bradford (1976), usando curva de albumina como padrão. Western Blotting foi realizado segundo Towbin (Towbin *et al.*, 1979). Alíquotas de cada amostra, com a mesma concentração de proteína total (45µg), foram submetidas à eletroforese em gel de poliacrilamida (Laemmli, 1970). Em seguida, as proteínas do gel foram transferidas eletricamente para membranas de nitrocelulose, por 6 horas a 50 V. Após a transferência, as membranas foram incubadas em solução bloqueadora (5% de leite desnatado em solução basal - Tris-HCl 10mM, NaCl 150mM e Tween 20 0,02%), overnight, à 4ºC. Em seguida, as membranas foram incubadas com anticorpo anti-UCP-1 em solução basal acrescida de 3% de leite desnatado, por 3 horas, à temperatura ambiente. Após as lavagens para remoção do excesso de anticorpo primario, as membranas foram incubadas com o

anticorpo secundario anti-IgG conjugado com a peroxidase, por 1 hora, em solução basal contendo leite a 1%, à temperatura ambiente. Após as lavagens (4 x 5 min), as membranas foram incubadas com o substrato para a peroxidase (Pierce ECL WB substrate, Thermo Scientific, USA) por 5 min e imediatamente expostas a filmes de raio-X por períodos variáveis de tempo, de 1 a 30 minutos. Os filmes foram revelados de forma convencional. As intensidades das bandas das auto-radiografias foram quantificadas por densitometria óptica, pelo programa Image J (NIH, Maryland, EUA). A normalização das bandas foi realizada usando o corante Pounceau S (Lagranha *et al.* 2010)

3.6 Análise estatística

Todos os dados foram analisados segundo a normalidade da distribuição. Os dados estiveram dentro da distribuição gaussiana e foram expressos em média e erro padrão da média (EPM). O teste *t student* foi utilizado para comparação entre médias de dois grupos. O Two-way ANOVA com múltiplas comparações foi utilizado para análises repetidas. Foi adotado o nível de significância quando $p \leq 0,05$. A construção do banco de dados e as análises estatísticas foram desenvolvidas no programa Excel (versão 2009, Microsoft, USA) e Graphpad Prisma 6® (GraphPad Software Inc., La Jolla, CA, USA), respectivamente.

4 RESULTADOS

Os resultados da pesquisa encontram-se apresentados em forma de artigo, os quais estão dispostos nos Apêndices:

A- TRATAMENTO COM FLUOXETINA EM RATOS NEONATOS REDUZ SIGNIFICANTEMENTE O ESTRESSE OXIDATIVO EM HIPOCAMPO E EM INDICADORES DE ANSIEDADE TARDIA NA VIDA PÓS-NATAL;

B- TRATAMENTO CRÔNICO COM FLUOXETINA E BIOENERGÉTICA MITOCONDRIAL EM TECIDO CENTRAL E PERIFÉRICO DE RATOS;

C- MANIPULAÇÃO NEONATAL DA SEROTONINA ALTERA O BALANÇO ENERGÉTICO: PARTICIPAÇÃO DAS MITOCÔNDRIAS E UCP EM TECIDO ADIPOSO MARROM.

5 CONSIDERAÇÕES FINAIS

As mitocôndrias são as principais fontes endógenas geradoras das espécies reativas de oxigênio sendo portanto**,** um possível alvo de drogas com potencial terapêutico ainda pouco explorado. Estudos prévios sugerem a possibilidade de que antidepressivos, estabilizadores de humor, e outros medicamentos, podem apresentar efeitos terapêuticos através de sua ação sobre as funções mitocondriais. Entretanto os efeitos da utilização dos ISRS durante períodos do desenvolvimento e sua ação sobre as mitocôndrias ainda são escassos. Em nosso estudo, observamos que os efeitos da administração crônica de fluoxetina em ratos neonatos promove significativa redução no crescimento somático dos animais sem diferenças significativas no consumo de alimentos e na atividade voluntária livre, sugerindo que a perda de peso ocorra devido a maior ativação do metabolismo mitocondrial tanto em tecidos centrais como periféricos, possivelmente tanto pelo aumento da dissipação de calor, como também pela maior quebra de nutrientes, confirmando nossas hipóteses. Podemos concluir com nossas investigações que o tratamento neonatal com fluoxetina em nosso modelo experimental promoveu uma modulação positiva em centros controladores do balanço energético sem causar prejuízos à função mitocondrial de células do tecido adiposo marrom, hipotálamo e músculo esquelético o que possivelmente reduziria o risco para o aparecimento e desenvolvimento de doenças metabólicas.

REFERÊNCIA BIBLIOGRÁFICA

ABDEL-RAZAQ, W.; KENDALL, D. A.; BATES, T. E. The effects of antidepressants on mitochondrial function in a model cell system and isolated mitochondria. **Neurochem Res**, v. 36, n. 2, p. 327-38, Feb 2011.

ABIZAID, A.; HORVATH, T. L. Ghrelin and the central regulation of feeding and energy balance. **Indian J Endocrinol Metab**, v. 16, n. Suppl 3, p. S617-26, Dec 2012.

ADAMS, S. H. Uncoupling protein homologs: emerging views of physiological function. **J Nutr**, v. 130, n. 4, p. 711-4, Apr 2000.

AEBI, H. Catalase in vitro. **Methods Enzymol**, v. 105, p. 121-6, 1984.

AHMAD, A. et al. Alterations in monoamine levels and oxidative systems in frontal cortex, striatum, and hippocampus of the rat brain during chronic unpredictable stress. **Stress**, v. 13, n. 4, p. 355-64, Jul 2010.

ALENINA, N. et al. Growth retardation and altered autonomic control in mice lacking brain serotonin. **Proc Natl Acad Sci** U S A, v. 106, n. 25, p. 10332-7, Jun 23 2009.

BELLINGER, L.; LILLEY, C.; LANGLEY-EVANS, S. C. Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat. **Br J Nutr**, v. 92, n. 3, p. 513-20, Sep 2004.

BENTO-SANTOS, A. et al. Criterios para escolha da amostra em experimentos com ratos Wistar. **RESBCAL**, v. 1, n. 1, p. 121-129, 2012.

BERGLUND, E. D. et al. Serotonin 2C receptors in pro-opiomelanocortin neurons regulate energy and glucose homeostasis. **J Clin Invest**, v. 123, n. 12, p. 5061-70, Dec 2 2013.

BLAKE, D. R.; WINYARD, P. G.; MAROK, R. The contribution of hypoxia-reperfusion injury to inflammatory synovitis: the influence of reactive oxygen intermediates on the transcriptional control of inflammation. **Ann N Y Acad Sci,** v. 723, p. 308-17, Jun 17 1994.

BLATTNER, J. R.; HE, L.; LEMASTERS, J. J. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. **Anal Biochem**, v. 295, n. 2, p. 220-6, Aug 15 2001.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal Biochem**, v. 72, p. 248-54, May 7 1976.

BRAND, M. D. et al. The significance and mechanism of mitochondrial proton conductance. **Int J Obes Relat Metab Disord,** v. 23 Suppl 6, p. S4-11, Jun 1999.

BRAND, M. D.; NICHOLLS, D. G. Assessing mitochondrial dysfunction in cells. **Biochem J,** v. 435, n. 2, p. 297-312, Apr 15 2011.

BRAND, M. D. et al. Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3. **Biochem J,** v. 368, n. Pt 2, p. 597-603, Dec 1 2002.

BRITO, M. N. et al. Differential activation of the sympathetic innervation of adipose tissues by melanocortin receptor stimulation. **Endocrinology,** v. 148, n. 11, p. 5339-47, Nov 2007.

BROBERGER, C. Brain regulation of food intake and appetite: molecules and networks. **J Intern Med**, v. 258, n. 4, p. 301-27, Oct 2005.

BRUINSTROOP, E. et al. Hypothalamic neuropeptide Y (NPY) controls hepatic VLDL-triglyceride secretion in rats via the sympathetic nervous system. **Diabetes**, v. 61, n. 5, p. 1043-50, May 2012.

BUEGE, J. A.; AUST, S. D. Microsomal lipid peroxidation. **Methods Enzymol,** v. 52, p. 302-10, 1978.

BUGGE, A.; DIB, L.; COLLINS, S. Measuring respiratory activity of adipocytes and adipose tissues in real time. **Methods Enzymol.** v.538, p. 233-47, 2014.

CASES, O. et al. Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: role of a serotonin excess during the critical period. **Neuron**, v. 16, n. 2, p. 297-307, Feb 1996.

CERRITO, F.; RAITERI, M. Evidence for an autoreceptor-mediated presynaptic control of serotonin release in central nerve endings [proceedings]. **Br J Pharmacol**, v. 67, n. 3, p. 424P-425P, Nov 1979.

CHA, S. H. et al. Inhibition of hypothalamic fatty acid synthase triggers rapid activation of fatty acid oxidation in skeletal muscle. **Proc Natl Acad Sci** U S A, v. 102, n. 41, p. 14557-62, Oct 11 2005.

CHA, S. H. et al. Hypothalamic malonyl-CoA triggers mitochondrial biogenesis and oxidative gene expression in skeletal muscle: Role of PGC-1alpha. **Proc Natl Acad Sci** U S A, v. 103, n. 42, p. 15410-5, Oct 17 2006.

CHORILLI, M.; MICHELIN, D. C.; SALGADO, H. R. N. Animais de laboratório: o camundongo. **Rev Ciênc Farm Básica**, v. 28, n. 1, p. 11-23, 2007.

CLAPHAM, J. C. et al. Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. **Nature,** v. 406, n. 6794, p. 415-8, Jul 27 2000.

CLARK, C. T.; WEISSBACH, H.; UDENFRIEND, S. 5-Hydroxytryptophan decarboxylase: preparation and properties. **J Biol Chem**, v. 210, n. 1, p. 139-48, Sep 1954.

CONE, R. D. et al. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. **Int J Obes Relat Metab Disord**, v. 25 Suppl 5, p. S63-7, Dec 2001.

COTE, F. et al. Maternal serotonin is crucial for murine embryonic development. **Proc Natl Acad Sci** U S A, v. 104, n. 1, p. 329-34, Jan 2 2007.

DA SILVA, A. I. et al. Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life. **Can J Physiol Pharmacol,** 2014.

DJORDJEVIC, J. et al. Fluoxetine affects antioxidant system and promotes apoptotic signaling in Wistar rat liver. **Eur J Pharmacol**, Mar 21 2011.

DLASKOVA, A.; CLARKE, K. J.; PORTER, R. K. The role of UCP 1 in production of reactive oxygen species by mitochondria isolated from brown adipose tissue. **Biochim Biophys Acta**, v. 1797, n. 8, p. 1470-6, Aug 2010.

ERSPAMER, V. Observations of the metabolism of endogenous 5-hydroxytryptamine (enteramine) in the rat. **Experientia**, v. 10, n. 11, p. 471-2, Nov 15 1954.

ERSPAMER, V. Historical introduction: the Italian contribution to the discovery of 5-hydroxytryptamine (enteramine, serotonin). **J Hypertens Suppl,** v. 4, n. 1, p. S3-5, Apr 1986.

FARIA, H. G. Conforto e bem estar dos animais de laboratório. **Arq. Ciênc. Saúde** Unipar, v. 2, n. 1, p. 83-87, 1998.

GARCIA-RUIZ, C. et al. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and

activation of transcription factor nuclear factor-kappa B: studies with isolated mitochondria and rat hepatocytes. **Mol Pharmacol,** v. 48, n. 5, p. 825-34, Nov 1995.

GEERLING, J. J. et al. Sympathetic nervous system control of triglyceride metabolism: novel concepts derived from recent studies. **J Lipid Res,** v. 55, n. 2, p. 180-9, Feb 2014.

GELLYNCK, E. et al. The serotonin 5-HT7 receptors: two decades of research. **Exp Brain Res**, v. 230, n. 4, p. 555-68, Oct 2013.

GREENWALD, R. A. Superoxide dismutase and catalase as therapeutic agents for human diseases. A critical review. **Free Radic Biol Med**, v. 8, n. 2, p. 201-9, 1990.

GRILL, H. J. Leptin and the systems neuroscience of meal size control. **Front Neuroendocrinol**, v. 31, n. 1, p. 61-78, Jan 2010.

GUTTERIDGE, J. M. Free radicals in disease processes: a compilation of cause and consequence. **Free Radic Res Commun**, v. 19, n. 3, p. 141-58, 1993.

GUYENET, S. J.; SCHWARTZ, M. W. Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the pathogenesis and treatment of obesity. **J Clin Endocrinol Metab**, v. 97, n. 3, p. 745-55, Mar 2012.

HABIG, W. H.; JAKOBY, W. B. Glutathione S-transferases (rat and human). **Methods Enzymol,** v. 77, p. 218-31, 1981.

HALFORD, J. C.; BLUNDELL, J. E. The 5-HT1B receptor agonist CP-94,253 reduces food intake and preserves the behavioural satiety sequence. **Physiol Behav**, v. 60, n. 3, p. 933-9, Sep 1996.

HALFORD, J. C.; WANNINAYAKE, S. C.; BLUNDELL, J. E. Behavioral satiety sequence (BSS) for the diagnosis of drug action on food intake. **Pharmacol Biochem Behav**, v. 61, n. 2, p. 159-68, Oct 1998.

HALLIWELL, B.; ARUOMA, O. I. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. **FEBS Lett,** v. 281, n. 1-2, p. 9-19, Apr 9 1991.

HEISLER, L. K. et al. Activation of central melanocortin pathways by fenfluramine. **Science**, v. 297, n. 5581, p. 609-11, Jul 26 2002.

HEISLER, L. K. et al. Serotonin reciprocally regulates melanocortin neurons to modulate food intake. **Neuron**, v. 51, n. 2, p. 239-49, Jul 20 2006.

HENDRICKS, T. J. et al. Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. **Neuron**, v. 37, n. 2, p. 233-47, Jan 23 2003.

HEPPLE, R. T. Mitochondrial involvement and impact in aging skeletal muscle. **Front Aging Neurosci**, v. 6, p. 211, 2014.

HIEMKE, C.; HARTTER, S. Pharmacokinetics of selective serotonin reuptake inhibitors. **Pharmacol Ther**, v. 85, n. 1, p. 11-28, Jan 2000.

HOYER, D. et al. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). **Pharmacol Rev,** v. 46, n. 2, p. 157-203, Jun 1994.

HOYER, D.; HANNON, J. P.; MARTIN, G. R. Molecular, pharmacological and functional diversity of 5-HT receptors. **Pharmacol Biochem Behav,** v. 71, n. 4, p. 533-54, Apr 2002.

HUGHES, J.; CRISCUOLO, F. Evolutionary history of the UCP gene family: gene duplication and selection. **BMC Evol Biol,** v. 8, p. 306, 2008.

IMAHASHI, K. et al. Transgenic expression of Bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. **Circ Res,** v. 95, n. 7, p. 734-41, Oct 1 2004.

INKIELEWICZ-STEPNIAK, I. Impact of fluoxetine on liver damage in rats. **Pharmacol Rep**, v. 63, n. 2, p. 441-7, 2011.

JEONG, J. K.; KIM, J. G.; LEE, B. J. Participation of the central melanocortin system in metabolic regulation and energy homeostasis. **Cell Mol Life Sci**, v. 71, n. 19, p. 3799-809, Oct 2014.

KEEN-RHINEHART, E.; ONDEK, K.; SCHNEIDER, J. E. Neuroendocrine regulation of appetitive ingestive behavior. **Front Neurosci,** v. 7, p. 213, 2013.

KHANZODE, S. D. et al. Oxidative damage and major depression: the potential antioxidant action of selective serotonin re-uptake inhibitors. **Redox Rep,** v. 8, n. 6, p. 365-70, 2003.

KIRKOVA, M. et al. Antioxidant activity of fluoxetine: studies in mice melanoma model. **Cell Biochem Funct,** v. 28, n. 6, p. 497-502, Aug 2010.

KRAUSS, S.; ZHANG, C. Y.; LOWELL, B. B. The mitochondrial uncoupling-protein homologues. **Nat Rev Mol Cell Biol,** v. 6, n. 3, p. 248-61, Mar 2005.

LAEMMLI, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, v. 227, n. 5259, p. 680-5, Aug 15 1970.

LAGRANHA, C. J. et al. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. **Circ Res**, v. 106, n. 11, p. 1681-91, Jun 11 2010.

LEITE, A. C. et al. Mitochondria generated nitric oxide protects against permeability transition via formation of membrane protein S-nitrosothiols. **Biochim Biophys Acta,** v. 1797, n. 6-7, p. 1210-6, Jun-Jul 2010.

LESCH, K. P.; WAIDER, J. Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. **Neuron,** v. 76, n. 1, p. 175-91, Oct 4 2012.

MALOYAN, A. et al. Mitochondrial dysfunction and apoptosis underlie the pathogenic process in alpha-B-crystallin desmin-related cardiomyopathy. **Circulation**, v. 112, n. 22, p. 3451-61, Nov 29 2005.

MARCONDES, F. K.; BIANCHI, F. J.; TANNO, A. P. Determination of the estrous cycle phases of rats: some helpful considerations. **Braz J Biol,** v. 62, n. 4A, p. 609-14, Nov 2002.

MAZER, C. et al. Serotonin depletion during synaptogenesis leads to decreased synaptic density and learning deficits in the adult rat: a possible model of neurodevelopmental disorders with cognitive deficits. **Brain Res**, v. 760, n. 1-2, p. 68-73, Jun 20 1997.

MEISTER, A.; ANDERSON, M. E. Glutathione. **Annu Rev Biochem**, v. 52, p. 711-60, 1983.

MILLER, B. H. et al. Genetic regulation of behavioral and neuronal responses to fluoxetine. **Neuropsychopharmacology,** v. 33, n. 6, p. 1312-22, May 2008.

MISRA, H. P.; FRIDOVICH, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. **J Biol Chem**, v. 247, n. 10, p. 3170-5, May 25 1972.

MORETTI, M. et al. Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress. **J Psychiatr Res**, v. 46, n. 3, p. 331-40, Mar 2012.

MORTON, G. J. et al. Central nervous system control of food intake and body weight. **Nature**, v. 443, n. 7109, p. 289-95, Sep 21 2006.

NASYROVA, D. I. et al. [Development of central and peripheral serotonin-producing systems in rats in ontogenesis]. Zh **Evol Biokhim Fiziol,** v. 45, n. 1, p. 68-74, Jan-Feb 2009.

NONOGAKI, K. et al. Altered gene expressions involved in energy expenditure in 5-HT(2C) receptor mutant mice. **Biochem Biophys Res Commun,** v. 295, n. 2, p. 249-54, Jul 12 2002.

PERSICO, A. M. et al. Barrel pattern formation requires serotonin uptake by thalamocortical afferents, and not vesicular monoamine release. **J Neurosci,** v. 21, n. 17, p. 6862-73, Sep 1 2001.

QU, Y. et al. Pharmacokinetics and pharmacodynamics of norfluoxetine in rats: Increasing extracellular serotonin level in the frontal cortex. **Pharmacol Biochem Behav**, v. 92, n. 3, p. 469-73, May 2009.

RAPPORT, M. M.; GREEN, A. A.; PAGE, I. H. Serum vasoconstrictor, serotonin; isolation and characterization. **J Biol Chem,** v. 176, n. 3, p. 1243-51, Dec 1948.

RODRIGUEZ, E. M.; BLAZQUEZ, J. L.; GUERRA, M. The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. **Peptides**, v. 31, n. 4, p. 757-76, Apr 2010.

ROSEN, G. M. et al. Free radicals and phagocytic cells. **FASEB J**, v. 9, n. 2, p. 200-9, Feb 1995.

SANCHEZ, S. et al. Circadian variations of serotonin in plasma and different brain regions of rats. **Mol Cell Biochem**, v. 317, n. 1-2, p. 105-11, Oct 2008.

SANDLER, M.; REVELEY, M. A.; GLOVER, V. Human platelet monoamine oxidase activity in health and disease: a review. **J Clin Pathol,** v. 34, n. 3, p. 292-302, Mar 1981.

SAVELIEVA, K. V. et al. Genetic disruption of both tryptophan hydroxylase genes dramatically reduces serotonin and affects behavior in models sensitive to antidepressants. **PLoS One**, v. 3, n. 10, p. e3301, 2008.

SEMPLE-ROWLAND, S. L.; DAWSON, W. W. Cyclic light intensity threshold for retinal damage in albino rats raised under 6 lx. **Exp Eye Res**, v. 44, n. 5, p. 643-61, May 1987.

SIES, H. Role of reactive oxygen species in biological processes. **Klin Wochenschr,** v. 69, n. 21-23, p. 965-8, Dec 15 1991.

SIES, H.; MEHLHORN, R. Mutagenicity of nitroxide-free radicals. **Arch Biochem Biophys**, v. 251, n. 1, p. 393-6, Nov 15 1986.

SILVA, J. P. et al. SOD2 overexpression: enhanced mitochondrial tolerance but absence of effect on UCP activity. **EMBO J**, v. 24, n. 23, p. 4061-70, Dec 7 2005.

SIMANSKY, K. J. Serotonergic control of the organization of feeding and satiety. **Behav Brain Res**, v. 73, n. 1-2, p. 37-42, 1996.

SULLIVAN, E. C.; MENDOZA, S. P.; CAPITANIO, J. P. Similarity in temperament between mother and offspring rhesus monkeys: sex differences and the role of monoamine oxidase-a and serotonin transporter promoter polymorphism genotypes. **Dev Psychobiol,** v. 53, n. 6, p. 549-63, Sep 2011.

TODA, C.; DIANO, S. Mitochondrial UCP2 in the central regulation of metabolism. **Best Pract Res Clin Endocrinol Metab,** v. 28, n. 5, p. 757-64, Oct 2014.

TOWBIN, H.; STAEHELIN, T.; GORDON, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. **Proc Natl Acad Sci** U S A, v. 76, n. 9, p. 4350-4, Sep 1979.

VAN ZUTPHEN, L. F. Toxicity testing and genetic quality control. **J Exp Anim Sci**, v. 35, n. 5-6, p. 202-9, Sep 1993.

VICKERS, M. H. et al. The effect of neonatal leptin treatment on postnatal weight gain in male rats is dependent on maternal nutritional status during pregnancy. **Endocrinology,** v. 149, n. 4, p. 1906-13, Apr 2008.

VOLPICELLI, F. et al. The serotonin receptor 7 and the structural plasticity of brain circuits. **Front Behav Neurosci**, v. 8, p. 318, 2014.

WILLIAMS, A. A. et al. Reduced levels of serotonin 2A receptors underlie resistance of Egr3-deficient mice to locomotor suppression by clozapine. **Neuropsychopharmacology**, v. 37, n. 10, p. 2285-98, Sep 2012.

WILLIAMS, K. W.; ELMQUIST, J. K. Lighting up the hypothalamus: coordinated control of feeding behavior. **Nat Neurosci**, v. 14, n. 3, p. 277-8, Mar 2011.

WONG, D. T.; BYMASTER, F. P. Development of antidepressant drugs. Fluoxetine (Prozac) and other selective serotonin uptake inhibitors. **Adv Exp Med Biol,** v. 363, p. 77-95, 1995.

WYNNE, K. et al. Appetite control. **J Endocrinol**, v. 184, n. 2, p. 291-318, Feb 2005.

ZAFIR, A.; ARA, A.; BANU, N. Invivo antioxidant status: a putative target of antidepressant action. **Prog Neuropsychopharmacol Biol Psychiatry**, v. 33, n. 2, p. 220-8, Mar 17 2009.

ZHENG, G. et al. Acute cold exposure and rewarming enhanced spatial memory and activated the MAPK cascades in the rat brain. **Brain Res**, v. 1239, p. 171-80, Nov 6 2008.

APÊNDICES

Apêndice A- ***"FLUOXETINE TREATMENT OF RAT NEONATES SIGNIFICANTLY REDUCES OXIDATIVE STRESS IN THE HIPPOCAMPUS AND IN BEHAVIORAL INDICATORS OF ANXIETY LATER IN POSTNATAL LIFE" foi submetido e aceito na revista Canadian Journal Physiology and Pharmacology (Qualis B1 em Nutrição).***

Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life

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Running title: High levels of serotonin during brain development period.

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ABSTRACT

The brain, more than any other organ in the body, is vulnerable to oxidative stress damage due to the its requirement for high levels of oxygenation to fulfill its metabolic needs in the face of relatively low levels of antioxidants to protect it. Recent studies suggest a direct involvement of oxidative stress in the etiology of eating and anxiety behaviors. The aim of this study was to evaluate the effect of fluoxetine-inhibited serotonin reuptake in nursing rat neonates, on behavior and on oxidative stress in the hypothalamus and the hippocampus, brain areas responsible for behavior related to food and anxiety, respectively. Results show that increased serotonin during a critical period of development does not induce significant differences in food-related behaviors (intake and satiety), but does result in a significant decrease in anxiety. Measurements of oxidative stress showed a significant reduction of lipid peroxidation in the hippocampus (57%). In hypothalamus, antioxidant enzymes were unchanged, but in hippocampus catalase and glutathione-S-transferase activity was increased (80% and 85%, respectively). Suggesting that protection of neural cells from oxidative stress during brain development could conceivably contribute to the anxiolytic effects of serotonin.

Keywords: Oxidative Stress; Anxiety; Serotonin; Fluoxetine

1. INTRODUCTION

Fluoxetine (commercially known as Prozac, Prozac Weekly, Sarafem, Rapiflux or Selfemra) is a serotonin reuptake inhibitor widely used for the treatment of depressive disorders in women during pregnancy and postpartum period (Wong and Bymaster 1995; Wong et al. 1995). According to some authors, this drug acts by increasing levels of extracellular serotonin in the brain's serotonergic system areas such as the frontal cortex and hippocampus, which may be involved in controlling emotion, memory and cognition (Krishnan and Nestler 2008). Fluoxetine is highly lipophilic, crosses the human placenta (Francis-Oliveira et al. 2013) and present in breast milk (Davanzo et al. 2011; Francis-Oliveira et al. 2013). Previous papers suggest that fluoxetine may exert harmful effects on developing fetuses and newborns to increase the risk of neurodevelopmental disorders and cause behavioral deficits (Nulman et al. 2002; Oberlander et al. 2009).

Furthermore, studies using experimental animals have shown that chronic treatment with fluoxetine in pregnant rats alters the pattern of hippocampal response to stress in their offspring (Olivier et al. 2011a; Olivier et al. 2011b; Pawluski et al. 2012). Serotonergic neurons in the brain and in the peripheral nervous system control numerous behaviors related to anxiety and food consumption (Blundell 1984; Green 1984; Iversen 1984). Recently fluoxetine and similar drugs have become a standard treatment for anxiety disorders (Baldwin et al. 2011; Crupi et al. 2011), and previous studies have shown that similar drugs (sertraline, fenfluramine) result in decreased food intake and body weight (Simansky and Vaidya 1990; Wellman et al. 2003).

Recent reports have shown that modulation of serotonin may also affect levels of reactive oxygen species (ROS) in the brain (Chung et al. 2010). The production of ROS exists essentially in balance with the production of antioxidant defense molecules. However, the balance is not perfect, so under some conditions ROS may come to dominate and promote cellular damage that could manifest itself in behavioral changes or neurodegenerative disease (Navarro and Boveris 2007; Trouche et al. 2010). Brain tissue is especially sensitive to oxidative damage since it engages in one of the highest levels of O_2 consumption in the body, although it accounts for only a small percentage of body weight, the brain is responsible for about 20% of basal oxygen consumption (Halliwell 2006). Sensitivity to ROS damage is also increased by the presence of excitotoxic amino acid neurotransmitters that generate superoxide and nitric oxide at the neural synapse and by autoxidizable neurotransmitters such as serotonin that react with superoxide to produce oxidized serotonin (Wrona and Dryhurst 1998).

Due to the nervous system vulnerability during pre- and post-natal periods the use of serotonin reuptake inhibitors may result in a change in behavior, which can cause injury to the adult offspring health. In association to the dual actions of serotonin as both a ROS-inhibitor and an anxiolytic and appetite-suppressing compound we decided to investigate whether the pharmacological manipulation of the serotonergic signaling system during a critical period of rat development could alter oxidative stress markers in brain areas that control feeding and anxiety behaviors. To this end, we used fluoxetine treatment during lactation to elevate serotonin levels, and then performed standardized tests for feeding- and anxiety- behaviors in conjunction with the evaluation of lipid peroxidation and antioxidant enzymes activity in both the hypothalamus and hippocampus of *Wistar* rats at 60 days of life.

2. MATERIALS AND METHODS

2.1 Animals

The animal-use protocols in this study have been approved by the Ethics Committee for Animal Research, at the Federal University of Pernambuco, in accordance with the guidelines published in "Principles of Laboratory Animal Care" (NIH, Bethesda, USA) and Canadian Council on Animal Care (CCAC) guidelines (Ethical Protocol 23076.026644/2010-20). Female Wistar rats (*Rattus norvegicus*) were maintained at a room temperature of 23 ± 1 °C, and in a 12-h alternating light–dark cycle (light 6:00 a.m. – 6:00 p.m.). Animals were housed collectively (8 females per cage during lactation), and the dams received standard laboratory chow *ad libitum*. After weaning, the rat pups were provided the same diet as their mothers, also *ad libidum*.

2.2 Pharmacological treatment and experimental groups

The offspring received once a day subcutaneous injection of fluoxetine (10 mg/kg, dissolved in saline solution, 10 ml/kg, bw; treated group) or vehicle (NaCl0.9%, 10 ml/kg, bw; control group) from the 1st to the 21st postnatal day (i.e., during the suckling period) (Silva et al. 2010). To avoid a possible influence of the circadian rhythm in these studies, injections were always administered between 7:00 a.m. and 8:00 a.m.

2.3 Measurement of body weight

Body weights were measured from 1st to the 21st postnatal day (weaning), and again at 50 and 60 days after birth (Mendes-da-Silva et al. 2002).

2.4 Behavioral Satiety Sequence (BSS)

Analysis of the behavioral satiety-sequence (BSS) was performed at 45 days of life was performed as described previously by Antin et al. (1975) (Antin et al. 1975; Oliveira Ldos et al. 2011; Orozco-Solis et al. 2009). The BSS measures the orderly transitions of eating, activity grooming and resting during the post-ingestive period. Using this analysis, we can determine:

1. Food intake: Food intake was measured by weighing the difference in the weight of the food bowl before, and a feeding period that followed 3h of food deprivation.
2. Microstructural analysis of behavior (bi-dimensional profiles): To analyze the change in behavior over time, data from a 60-min continuous record of behavior in each animal can be divided into 5-min periods. The true duration of each individual behavior in each period is calculated. Profiles are plotted for each behavior over the 60-min period.
3. Interpreting the BSS: Behaviors were categorized as: eating (ingesting food, gnawing, chewing or holding food in paws), drinking, an active state (exploratory movements around the cage; rearing), grooming (body care movements performed with the mouth or forelimbs), and a resting state (sitting or lying in a resting position; sleeping animal).

2.5 Elevated plus-maze (EPM) test

The EPM test was performed at 55 days of life (N = 10 in each group at each time). The EPM test is widely used to observe behaviors relevant to understanding anxiety in animal studies (Drapier et al. 2007; Lister 1987). The EPM apparatus consists of two open arms (50 x 10 x 1 cm) and two enclosed arms (50 x 10 x 40 cm) originating from a common central platform (10 x 10 cm) to form a plus shape. The entire apparatus was elevated to a height of 50 cm above the floor. A video camera and illumination lamps were mounted on the ceiling. The anxiety-like behavior of each animal were recorded for a period of 5 min by a VCR-recording system. At the beginning of the test, the rat was placed on the central platform with its head facing an open arm. The arm entry was defined as all four paws into either an open or a closed arm. The total time each animal spent in various sections of the maze (open arms, center, or enclosed arms) was recorded. The results are reported as time spent in open arms and number of entries of open arms.

2.6 Biochemical Analysis

For biochemical analyzes, 60-day old rats were first anesthetized (ketamine:xylazine, 60:5 mg/kg, i.p) and then challenged (verification of foot reflex; since significant number of rodents do not lose the foot reflex with anesthetics, decapitation was performed 10 minutes after injection, time to surgical anesthesia occurs), in order to verify whether anesthesia was reached before decapitation. The brains regions containing the hippocampus and hypothalamus were rapidly dissected and saved at -80°C for later analysis. For the biochemical experiments the tissues were homogenized in Tris-EDTA buffer (Tris 100 mM, pH 7.5; EDTA 10 mM and protease inhibitors) on ice and centrifuged for 10 min at 5.000 x g at 4 °C. Aliquots of supernatant were analyzed for total protein content using the Bradford protocol (Bradford 1976).

2.6.1 Evaluation of Lipid Peroxidation

The tissue MDA (malondialdehyde) level was determined based on its reaction with thiobarbituric acid (TBA) at 100 °C according to Draper et al. (1993) (Draper et al. 1993). In the TBA test reaction, MDA or MDA-like substances react with TBA to produce a pink pigment with an absorption maximum of 535 nm. Briefly, the reaction was developed by the sequential addition of TBA in combination with sample, 30% trichloroacetic acid and Tris-HCl, mixed thoroughly and centrifuged at 2.500 g for 10 min. After centrifugation the supernatant was transferred to another tube and 0,8% TBA (v/v) was added, before mixing and boiling in a water-bath for 30 min. After cooling in tap water, n-butanol was added and the absorbance at 535 nm of the organic phase was determined in a spectrophotometer. Results were expressed as nmol per mg of protein.

2.6.2 Superoxide dismutase activity

The determination of total SOD enzyme activity (t-SOD) was performed according to the method of Misra and Fridovich (1972) (Misra and Fridovich 1972). Samples were incubated in with sodium carbonate buffer (0.05%, pH 10.2, 0.1mM de EDTA) in a water bath at 37^{o}C. The reaction was started by addition of 30 mM epinephrine (in 0.05% acetic acid). Absorbance at 480 nm was determined for 4 min. One unit of t-SOD was defined as the enzyme amount causing 50% inhibition of epinephrine oxidation. Tissue t-SOD activity was also expressed as units per milligram protein (U/ mg protein).

2.6.3 Catalase activity

Catalase activity was measured according to Aebi (1984), based on the determination of the rate constant, k, of H_2O_2 decomposition. Under our conditions of temperature and pH, k was defined as 4.6 $x10^7$. By measuring the absorbance changes per minute for 4 min, the rate constant of the enzyme was determined. Enzyme activities were expressed as H_2O_2 consumed per unit time per amount of protein (nM/min/mg protein) (Aebi 1984).

2.6.4 Glutathione S-transferases activity

Glutathione S Transferase (GST) is an antioxidant enzyme involved in the detoxification of a wide range of toxic agents including peroxide and alkylating agents present in the brain. The activity of GST was measured by the method described by Habig et al. (1974) (Habig et al. 1974). The principle of the assay is based on the determination through absorbance spectroscopy of the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). Absorbance is measured at 340nm. One unit of enzyme conjugates 10.0 nmol of CDNB with reduced glutathione per minute.

2.7 Statistical analysis

All results are expressed as means ± SEM. The student t-test was used to assess the significant differences between the two groups. For the repeated analysis we use two-way ANOVA with multiple comparisons (i.e body weight analysis). Data were considered as statistically significant at a value of $p \leq 0.05$. All data were plotted and the statistical analysis performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS

3.1 Body weight (bw) evaluation

Since the fluoxetine treatment was maintained throughout nursing period in the offspring we measured the body weight daily in order to assess whether high levels of serotonin could alter growth and development compared to control. We observed that in the treated group, body weight was significantly lower than control beginning with the first week of life (Figure 1A). Furthermore, the body weight of the treated group remained lower than that of the control rats until the 60^{th} days of life (39 days after last drug application) (Figure 1B).

3.2 Food ingestion test

As a measure of the effect of the serotonin on eating behavior, we determined the amount of food intake during a 24 hour period. Surprisingly, given the decreased BW in the fluoxetine-treated group, 24 h food intake was not altered by the drug, suggesting that weight differences between the groups were not related to food intake (Control: 2.76 ± 0.19 g/ b.w *100 *vs.*Treated: 2.85 ± 0.26 g/b.w*100) (Figure 2).

3.3 Behavioral Satiety Sequence (BSS) evaluation

Several studies have suggested that the behavioral satiety sequence better represents rat behaviors on food consumption than other testing methods. Using BSS, we analyzed the behavioral satiety sequence during one hour, following three hours of fasting. Our data are summarized in Figure 3 and Table 1. We observed that the fluoxetine-treated group did not show a significant alteration in BSS patterns compared to control, although in microstructure analysis of behavior, we observed a significant difference in grooming duration and a trend toward increased resting duration in the treated group (Table 1).

3.4 Elevated plus-maze (EPM) test

After our evaluation of the impact of food-related behavior on body weight changes, we studied the influence of anxiety-like behavior on body weight, since levels are known to influence the demand for food. The fluoxetine-increased levels of serotonin during the nursing period increased the number of entries in the open arms of the elevated plus-maze (Control= 8.7 $\pm$ 1.2; Treated= 11.6 $\pm$ 0.20; p=0.0299). In addition, the treated animals showed a significant increase in the amount of time spent in the open arms of the elevated plus-maze (Control= 89.6 $\pm$ 14.1 seconds; Treated= 124.5 $\pm$ 5.4 seconds; p=0.0345). In association to the increase in entries and time spent in the open arms, fluoxetine treatment increase the percentage of open arm entries (Control= 43%; Treated= 48%; p=0.0489) and the percentage of time on the open arms (Control= 45%; Treated= 55% p=0.0158), together with decrease in time spent in close arms (Control= 148 $\pm$ 2.3 seconds; Treated= 109 $\pm$ 8 seconds; p=0.0042). Together, these results suggest that fluoxetine-treated group exhibited lower levels of anxiety-like behavior than the control group (Figure 4).

3.5 Effect of fluoxetine on MDA levels in hippocampus and hypothalamus

Due to their involvement in eating and anxiety-like behavior, respectively, the hypothalamus and hippocampus were examined for changes in lipid peroxidation occurring

with fluoxetine treatment. Increased levels of serotonin occurring with fluoxetine treatment during the nursing period appeared to induce divergent effects in these two regions. Although a significant reduction in lipid peroxidation was detected in the hippocampus of the treated group (Treated: 2.04 ± 0.1 nmol/mg prot; $p < 0.01$) compared to the control (Control: 6.04 ± 0.5 nmol/mg prot), and an increase was found in the hypothalamus (Control= 18.75 ± 1. 5 nmol/mg prot; Treated= 25.0 ± 0.75 nmol/mg prot) (Figure 5A and B).

3.6 Effect of fluoxetine treatment on antioxidant enzyme activities in the hippocampus and hypothalamus

High levels of lipid peroxidation can induce cell damage and/or cell death. However, most cells possess a protective mechanism to decrease the production of reactive oxygen species through the action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and/or glutathione S-transferase (GST). Therefore, we sought to examine whether the suppression of lipid peroxidation with fluoxetine treatment involved changes in the activity of any of these three enzymes in the hypothalamus or hippocampus. Results showed that fluoxetine treatment did not alter SOD activity in either the hypothalamus or hippocampus (hypothalamus: Control = 2.07 ± 0.22 U/mg prot; Fluoxetine = 1.412 ± 0.365 U/mg prot; hippocampus: Control = 1.372 ± 0.118 U/mg prot; Fluoxetine = 1.276±0.157 U/mg prot). In hippocampus, catalase activity was increased significantly by fluoxetine (Control = 2.5 ± 0.34nmol/min/mg prot; Fluoxetine = 4.124 ± 0.42 nmol/min/mg prot; $p < 0.01$); in contrast in hypothalamus the treatment wasn't induce significant difference (Control = 3.35 ± 0.59 nmol/min/mg prot; Fluoxetine = 3.93 ± 0.45 nmol/min/mg prot) when compared to control. Changes in GST activity, however, were similar to those observed in SOD and CAT activity, showing no significant change in activity with fluoxetine in the hypothalamus (Control=3.5 ± 0.68 mmol/mg prot; Fluoxetine = 5.25 ± 0.75 mmol/mg prot); although in hippocampus fluoxetine-treatment induce a significant increase in the enzymatic activity (Control = 2.81 ± 0.21 mmol/mg prot; Fluoxetine = 5.21 ±0.36 mmol/mg prot) (Figure 5C-H).

4. DISCUSSION

To our knowledge there have been no previous studies that examine both the effect of chronic elevated serotonin during rat development on lipid peroxidation and antioxidants in brain regions controlling eating behavior and anxiety (hypothalamus, and hypothalamus/hippocampus, respectively) and the eating and anxiety behaviors that occur in

the same set of treated animals. In the present study, we have used fluoxetine treatment of rats during nursing, a critical period in brain development, to assess possible relationships between effects of serotonin on reactive oxygen species in particular brain regions, and on behaviors associated with these regions. Our results show that increased levels of serotonin early in life in rats reduces body weight gain and anxiety levels and also lowers lipid peroxidation and modulates specific antioxidant enzymes in the hypothalamus and hippocampus and even 39 days after discontinuation of fluoxetine. These findings suggest that manipulation of the serotonergic system during critical periods of development can result in persistent changes in brain cell biochemical activities.

Our experimental model demonstrates a fluoxetine-induced reduction in body weight without incurring significant alteration of either food intake or behavior satiety sequence. These data corroborate earlier results from our research group using the same experimental model (Mendes-da-Silva et al. 2002). They contrast, however, with results of a recent study of serotonin up-regulation by tryptophan (L-Trp) in which the authors observed that L-Trp treatment induced an increase in body weight in association with the increasing hypothalamic production of serotonin (Shen et al. 2012). The discrepancy between our results and those of Shen et al. could be related to the method used to increase serotonin used in each (tryptophan vs, fluoxetine), or it may be due to the different species used in the studies; rats in the present work and in Mendes-da Silva's work, and pigs in the study of Shen et al.

Despite showing a decrease in body weight with fluoxetine, the present study could demonstrate no difference in food intake between the groups, in agreement with the results of previous study showing that different concentrations of fluoxetine in male rats from the 4th to 21st postnatal day produced no significant changes in food intake (Ansorge et al. 2004). Further studies (metabolic, biochemical) will be necessary to understand the mechanisms underlying the fluoxetine-induced reduction in body weight that occurs during the nursing period without measurably affecting food consumption.

Recent studies have pointed to a hypothesis that changes in specific behaviors are linked to the production of reactive oxygen species and the induction of oxidative stress in areas of the brain controlling those behaviors (El-Ansary et al. 2010; Gigante et al. 2011). Reactive oxygen species are already well known role as signaling molecules that control metabolism and feeding through their involvement in specific intracellular pathways (Diano et al. 2011; El-Ansary et al. 2010; Leloup et al. 2006). Leloup et al. (2006) demonstrated that ROS act activate intracellular signaling pathways in the hypothalamus that exercise metabolic control over glucose and lipids. In the present study we observed an increase in catalase

activity in the hippocampus and glutathione S-transferase activity, a decrease in lipid peroxidation, but no difference in superoxide dismutase. Benani et al. showed an association between oxidative stress and eating control is essential for the reduction of food intake observed in rats in stressful environments (Benani et al. 2007). We speculate that in our fluoxetine-treated rat model, the increase in antioxidant activity with serotonin up-regulation decrease the oxidative stress leading to changes in anxiety level, but insufficient to affect food intake. Previous investigations have attributed to fluoxetine an antioxidant role in a number of specific areas of the brain in both rats and humans (Moretti et al. 2012; Novio et al. 2011; Zafir et al. 2009) in parallel with the ability of fluoxetine to increase serotonin levels.

Ours results have also shown a reduction in level of anxiety as measured by the elevated plus-maze, occurring with an increase in hippocampal catalase and glutathione S-transferase activity, but with no difference in superoxide dismutase between control and fluoxetine-treated groups. The molecular mechanisms involved in the producing normal and pathological anxiety states are complex, and not totally understood. Recent studies have demonstrated a direct involvement of oxidative stress in producing anxiety (de Oliveira et al. 2007; Souza et al. 2007). Hovatta et al. (2005), studying the genesis of anxiety, observed a change in the expression of two genes associated with oxidative stress, glyoxalase 1 and glutathione reductase 1, and lending support to causative role of oxidative stress in the pathogenesis of this disease (Hovatta et al. 2005). Another study, using mice transgenic for a specific protein related to oxidative stress (p66Shc) found that the maintenance of low levels of oxidative stress may be able to prevent some of the behavioral effects of aging, in particular, the response to emotionally exciting stimuli. The authors concluded that complex interactions between oxidative stress and emotional stress might lie at the root of persistent changes in anxiety behavior (Berry et al. 2007).

In an another study involving oxidative stress and anxiety, Masood et al. (2008) demonstrated that when mice were treated with the pro-oxidative agent, L-buthionine-(S, R)-sulfoximine (BSO) and assessed using the elevated plus-maze, they demonstrated a reduction in of entries into open arms, as well as a reduction in the time spent in open arms compared with the control. In addition, when the mice were treated with BSO in combination with antioxidant agents (Bay 60-7550 and apocynin), the mice showed an increase both in entries into open arms and in the time spent in the open arms compared with BSO-treated only (Masood et al. 2008). Taking our current data together with literature on anxiety and oxidative stress, we suggest that modulation of serotonin levels by fluoxetine decreases levels of oxidative stress, and is, in turn, accompanied by a reduction in anxiety levels. Such results

suggest that early intervention with serotonin re-uptake inhibitors (like fluoxetine in the current study) could contribute to some of the anxiolytic effects of the drug, perhaps by altering neural function with reductions in oxidative stress. Further studies are warranted to fully comprehend how the modulation of oxidative stress by serotonin produces persistent effects on brain function and behavior state.

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REFERENCES:

Aebi, H. 1984. Catalase in vitro. Methods Enzymol *105*, 121-126.

Ansorge, M. S., Zhou, M., Lira, A., Hen, R., and Gingrich, J. A. 2004. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science *306*, 879-881.

Antin, J., Gibbs, J., Holt, J., Young, R. C., and Smith, G. P. 1975. Cholecystokinin elicits the complete behavioral sequence of satiety in rats. J Comp Physiol Psychol *89*, 784-790.

Baldwin, D., Woods, R., Lawson, R., and Taylor, D. 2011. Efficacy of drug treatments for generalised anxiety disorder: systematic review and meta-analysis. BMJ *342*, d1199.

Benani, A., Troy, S., Carmona, M. C., Fioramonti, X., Lorsignol, A., Leloup, C., Casteilla, L., and Penicaud, L. 2007. Role for mitochondrial reactive oxygen species in brain lipid sensing: redox regulation of food intake. Diabetes *56*, 152-160.

Berry, A., Capone, F., Giorgio, M., Pelicci, P. G., de Kloet, E. R., Alleva, E., Minghetti, L., and Cirulli, F. 2007. Deletion of the life span determinant p66Shc prevents age-dependent increases in emotionality and pain sensitivity in mice. Exp Gerontol *42*, 37-45.

Blundell, J. E. 1984. Serotonin and appetite. Neuropharmacology *23*, 1537-1551.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Chung, Y. C., Kim, S. R., and Jin, B. K. 2010. Paroxetine prevents loss of nigrostriatal dopaminergic neurons by inhibiting brain inflammation and oxidative stress in an experimental model of Parkinson's disease. J Immunol *185*, 1230-1237.

Crupi, R., Marino, A., and Cuzzocrea, S. 2011. New therapeutic strategy for mood disorders. Curr Med Chem *18*, 4284-4298.

Davanzo, R., Copertino, M., De Cunto, A., Minen, F., and Amaddeo, A. 2011. Antidepressant drugs and breastfeeding: a review of the literature. Breastfeed Med *6*, 89-98.

de Oliveira, M. R., Silvestrin, R. B., Mello, E. S. T., and Moreira, J. C. 2007. Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: effects of sub acute vitamin A supplementation at therapeutic doses. Neurotoxicology *28*, 1191-1199.

Diano, S., Liu, Z. W., Jeong, J. K., Dietrich, M. O., Ruan, H. B., Kim, E., Suyama, S., Kelly, K., Gyengesi, E., Arbiser, J. L., *et al.* 2011. Peroxisome proliferation-associated control of

reactive oxygen species sets melanocortin tone and feeding in diet-induced obesity. Nat Med *17*, 1121-1127.
Draper, H. H., Squires, E. J., Mahmoodi, H., Wu, J., Agarwal, S., and Hadley, M. 1993. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radic Biol Med *15*, 353-363.
Drapier, D., Bentue-Ferrer, D., Laviolle, B., Millet, B., Allain, H., Bourin, M., and Reymann, J. M. 2007. Effects of acute fluoxetine, paroxetine and desipramine on rats tested on the elevated plus-maze. Behav Brain Res *176*, 202-209.
El-Ansary, A., Al-Daihan, S., Al-Dbass, A., and Al-Ayadhi, L. 2010. Measurement of selected ions related to oxidative stress and energy metabolism in Saudi autistic children. Clin Biochem *43*, 63-70.
Francis-Oliveira, J., Ponte, B., Barbosa, A. P., Verissimo, L. F., Gomes, M. V., Pelosi, G. G., Britto, L. R., and Moreira, E. G. 2013. Fluoxetine exposure during pregnancy and lactation: Effects on acute stress response and behavior in the novelty-suppressed feeding are age and gender-dependent in rats. Behav Brain Res *252*, 195-203.
Gigante, A. D., Young, L. T., Yatham, L. N., Andreazza, A. C., Nery, F. G., Grinberg, L. T., Heinsen, H., and Lafer, B. 2011. Morphometric post-mortem studies in bipolar disorder: possible association with oxidative stress and apoptosis. Int J Neuropsychopharmacol *14*, 1075-1089.
Green, A. R. 1984. 5-HT-mediated behavior. Animal studies. Neuropharmacology *23*, 1521-1528.
Habig, W. H., Pabst, M. J., and Jakoby, W. B. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem *249*, 7130-7139.
Halliwell, B. 2006. Oxidative stress and neurodegeneration: where are we now? J Neurochem *97*, 1634-1658.
Hovatta, I., Tennant, R. S., Helton, R., Marr, R. A., Singer, O., Redwine, J. M., Ellison, J. A., Schadt, E. E., Verma, I. M., Lockhart, D. J., and Barlow, C. 2005. Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice. Nature *438*, 662-666.
Iversen, S. D. 1984. 5-HT and anxiety. Neuropharmacology *23*, 1553-1560.
Krishnan, V., and Nestler, E. J. 2008. The molecular neurobiology of depression. Nature *455*, 894-902.
Leloup, C., Magnan, C., Benani, A., Bonnet, E., Alquier, T., Offer, G., Carriere, A., Periquet, A., Fernandez, Y., Ktorza, A. 2006. Mitochondrial reactive oxygen species are required for hypothalamic glucose sensing. Diabetes *55*, 2084-2090.
Lister, R. G. 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) *92*, 180-185.
Masood, A., Nadeem, A., Mustafa, S. J., and O'Donnell, J. M. 2008. Reversal of oxidative stress-induced anxiety by inhibition of phosphodiesterase-2 in mice. J Pharmacol Exp Ther *326*, 369-379.
Mendes-da-Silva, C., de Souza, S. L., Barreto-Medeiros, J. M., de Freitas-Silva, S. R., Antunes, D. E., Cunha, A. D., Ribas, V. R., de Franca, M. F., Nogueira, M. I., and Manhaes-de-Castro, R. 2002. Neonatal treatment with fluoxetine reduces depressive behavior induced by forced swim in adult rats. Arq Neuropsiquiatr *60*, 928-931.
Misra, H. P., and Fridovich, I. 1972. The generation of superoxide radical during the autoxidation of hemoglobin. J Biol Chem *247*, 6960-6962.
Moretti, M., Colla, A., de Oliveira Balen, G., Dos Santos, D. B., Budni, J., de Freitas, A. E., Farina, M., and Severo Rodrigues, A. L. 2012. Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress. J Psychiatr Res *46*, 331-340.

Navarro, A., and Boveris, A. 2007. The mitochondrial energy transduction system and the aging process. Am J Physiol Cell Physiol *292*, C670-686.
Novio, S., Nunez, M. J., Amigo, G., and Freire-Garabal, M. 2011. Effects of fluoxetine on the oxidative status of peripheral blood leucocytes of restraint-stressed mice. Basic Clin Pharmacol Toxicol *109*, 365-371.
Nulman, I., Rovet, J., Stewart, D. E., Wolpin, J., Pace-Asciak, P., Shuhaiber, S., and Koren, G. 2002. Child development following exposure to tricyclic antidepressants or fluoxetine throughout fetal life: a prospective, controlled study. Am J Psychiatry *159*, 1889-1895.
Oberlander, T. F., Gingrich, J. A., and Ansorge, M. S. 2009. Sustained neurobehavioral effects of exposure to SSRI antidepressants during development: molecular to clinical evidence. Clin Pharmacol Ther *86*, 672-677.
Oliveira Ldos, S., da Silva, L. P., da Silva, A. I., Magalhaes, C. P., de Souza, S. L., and de Castro, R. M. 2011. Effects of early weaning on the circadian rhythm and behavioral satiety sequence in rats. Behav Processes *86*, 119-124.
Olivier, J. D., Blom, T., Arentsen, T., and Homberg, J. R. 2011a. The age-dependent effects of selective serotonin reuptake inhibitors in humans and rodents: A review. Prog Neuropsychopharmacol Biol Psychiatry *35*, 1400-1408.
Olivier, J. D., Valles, A., van Heesch, F., Afrasiab-Middelman, A., Roelofs, J. J., Jonkers, M., Peeters, E. J., Korte-Bouws, G. A., Dederen, J. P., Kiliaan, A. J., *et al.* 2011b. Fluoxetine administration to pregnant rats increases anxiety-related behavior in the offspring. Psychopharmacology (Berl) *217*, 419-432.
Orozco-Solis, R., Lopes de Souza, S., Barbosa Matos, R. J., Grit, I., Le Bloch, J., Nguyen, P., Manhaes de Castro, R., and Bolanos-Jimenez, F. 2009. Perinatal undernutrition-induced obesity is independent of the developmental programming of feeding. Physiol Behav *96*, 481-492.
Pawluski, J. L., Rayen, I., Niessen, N. A., Kristensen, S., van Donkelaar, E. L., Balthazart, J., Steinbusch, H. W., and Charlier, T. D. 2012. Developmental fluoxetine exposure differentially alters central and peripheral measures of the HPA system in adolescent male and female offspring. Neuroscience *220*, 131-141.
Shen, Y. B., Voilque, G., Kim, J. D., Odle, J., and Kim, S. W. 2012. Effects of elevating tryptophan intake on growth and physiological changes in nursery pigs. J Anim Sci.
Silva, C. M., Goncalves, L., Manhaes-de-Castro, R., and Nogueira, M. I. 2010. Postnatal fluoxetine treatment affects the development of serotonergic neurons in rats. Neurosci Lett *483*, 179-183.
Simansky, K. J., and Vaidya, A. H. 1990. Behavioral mechanisms for the anorectic action of the serotonin (5-HT) uptake inhibitor sertraline in rats: comparison with directly acting 5-HT agonists. Brain Res Bull *25*, 953-960.
Souza, C. G., Moreira, J. D., Siqueira, I. R., Pereira, A. G., Rieger, D. K., Souza, D. O., Souza, T. M., Portela, L. V., and Perry, M. L. 2007. Highly palatable diet consumption increases protein oxidation in rat frontal cortex and anxiety-like behavior. Life Sci *81*, 198-203.
Trouche, E., Mias, C., Seguelas, M. H., Ordener, C., Cussac, D., and Parini, A. 2010. Characterization of monoamine oxidases in mesenchymal stem cells: role in hydrogen peroxide generation and serotonin-dependent apoptosis. Stem Cells Dev *19*, 1571-1578.
Wellman, P. J., Jones, S. L., and Miller, D. K. 2003. Effects of preexposure to dexfenfluramine, phentermine, dexfenfluramine-phentermine, or fluoxetine on sibutramine-induced hypophagia in the adult rat. Pharmacol Biochem Behav *75*, 103-114Wong, D. T., and Bymaster, F. P. 1995. Development of antidepressant drugs. Fluoxetine (Prozac) and other selective serotonin uptake inhibitors. Adv Exp Med Biol *363*, 77-95.

Wong, D. T., Bymaster, F. P., and Engleman, E. A. 1995. Prozac (fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. Life Sci *57*, 411-441.
Wrona, M. Z., and Dryhurst, G. 1998. Oxidation of serotonin by superoxide radical: implications to neurodegenerative brain disorders. Chem Res Toxicol *11*, 639-650.
Zafir, A., Ara, A., and Banu, N. 2009. Invivo antioxidant status: a putative target of antidepressant action. Prog Neuropsychopharmacol Biol Psychiatry *33*, 220-228.

LEGENDS:

Figure 1-Effect of modulation of serotonin system during critic period of development on Body Weight. The values are from at least six rats of each group and expressed as means ± S.E.M. (*p<0.05).

Figure 2- Effect of modulation of serotonin system during critic period of development on food intake. The values are from at least six rats of each group and expressed as means ± S.E.M

Figure 3- Effect of modulation of serotonin system during critic period of development on Behavioral Satiety Sequence test. The values are from at least six rats of each group and expressed as means ± S.E.M. A) Control Group; B) Fluoxetine Group

Figure 4- Effect of modulation of serotonin system during critic period of development on anxiety-like behavior. The values are from at least six rats of each group and expressed as means ± S.E.M (*p<0.05); (**p<0.01). A) Time that the groups spent in open arms; B) Numbers of entries in open arm; C) Percentage of time spent on open arms; D) Percentage of entries in open arms; E) Time that the group spent in close arms; F) Percentage of entries on the close arms.

Figure 5-Effect of modulation of serotonin system related to oxidative stress in the hypothalamus and hippocampus. The values are from at least six rats of each group and expressed as means ± S.E.M. (*p<0.05); (**p<0.01); (***p<0.001).

Table 1: Effect of chronic fluoxetine treatment on microstructure of behavior. The values are from at least six rats of each group and expressed as means ± S.E.M. (*p<0.05); (**p<0.01)

FIGURES:

	Control	Fluoxetine
Eating duration (s)	1321.86±94.76	1132.56±78.20
Grooming duration (s)	80.0±30.92	209.69±88.88**
Resting duration (s)	1433.0±289.21	1572.44±143.22*
Locomotion duration (s)	781.43±146.79	732.78±98.36

Note: The values are from at least 6 rats per group; values are the mean ± SEM; *, $p < 0.05$ and **, $p < 0.01$ compared with the control group.

FIGURE 1)

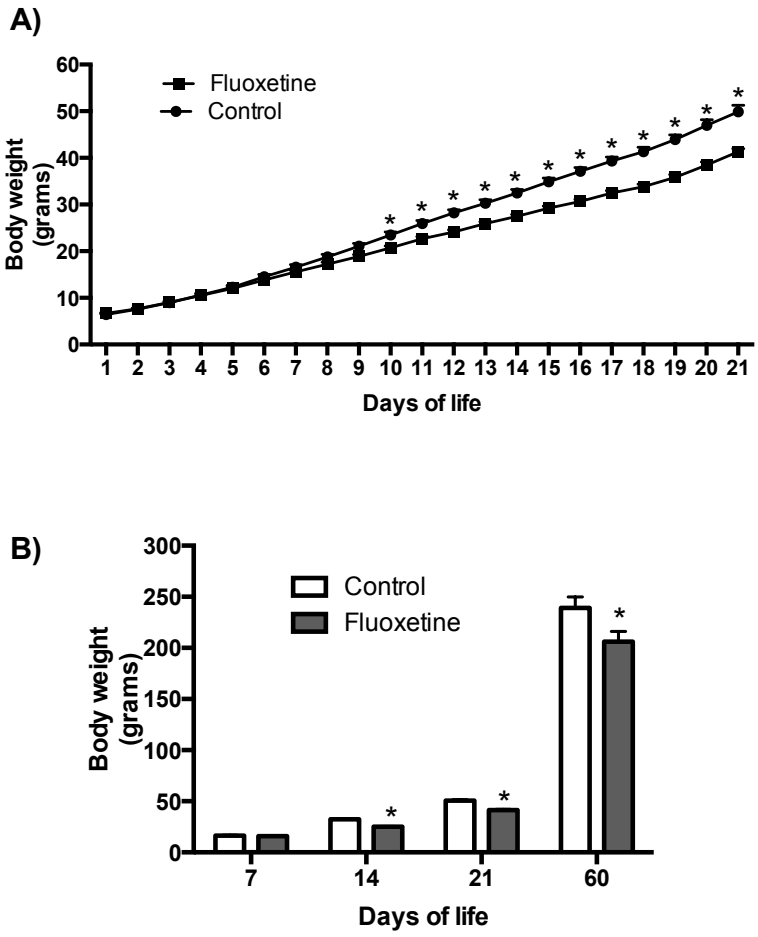


FIGURE 2)

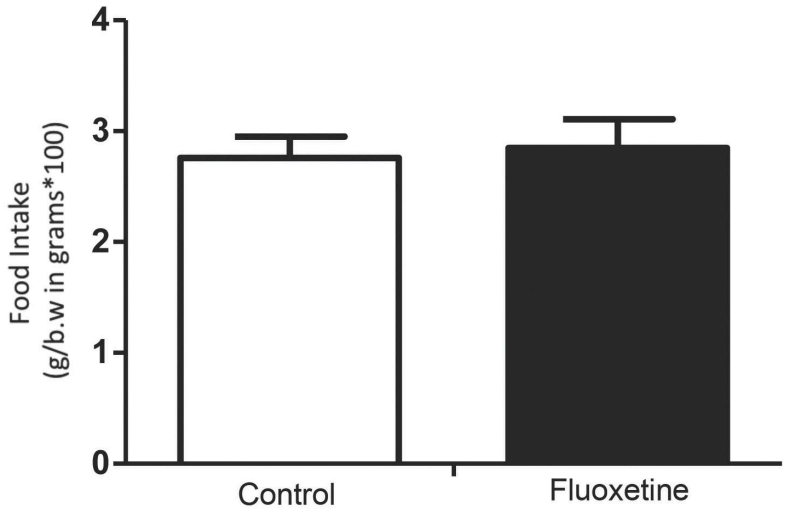


FIGURE 3)

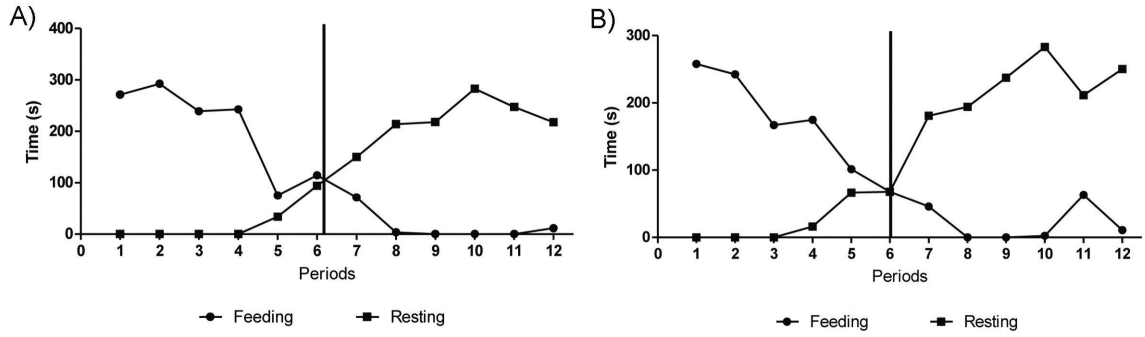


FIGURE 4)

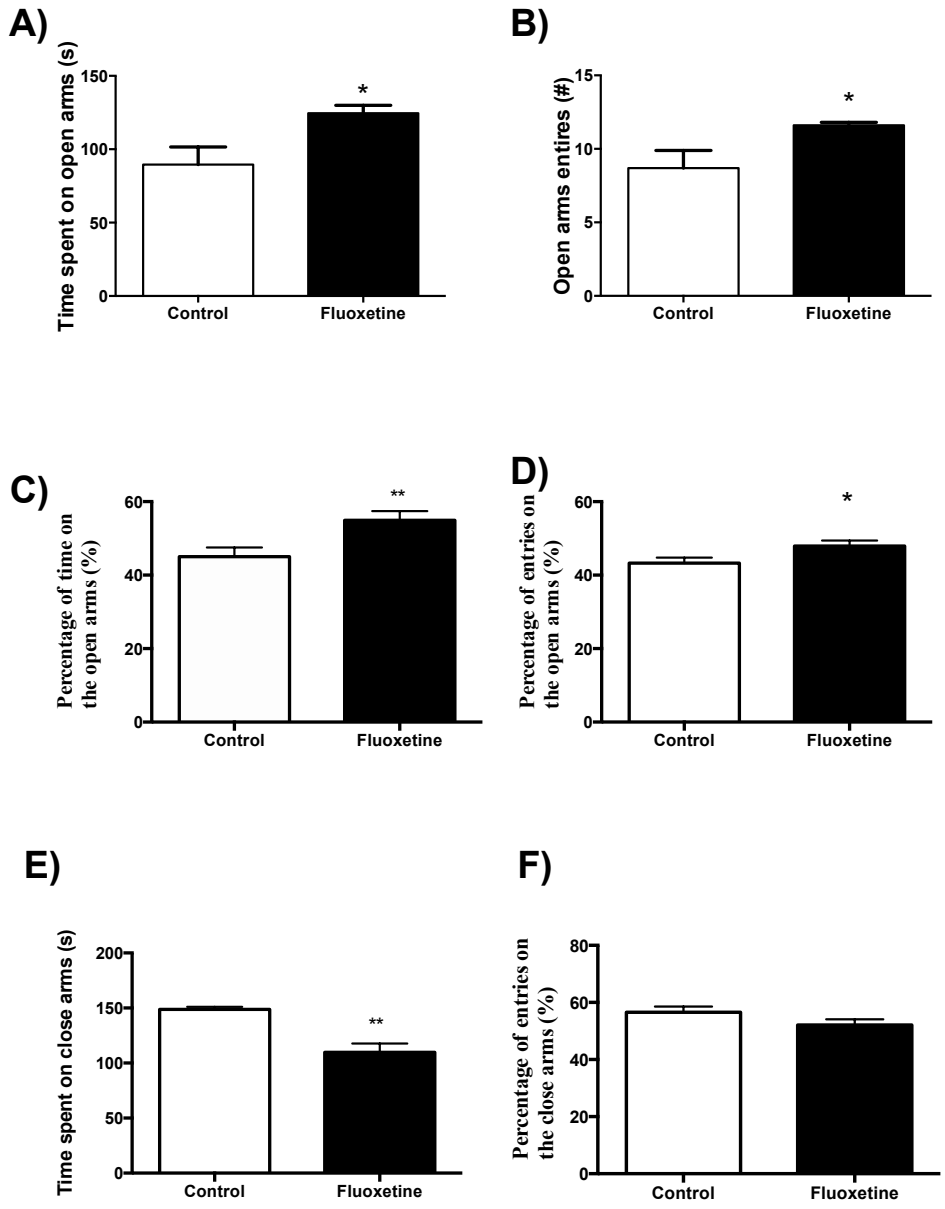
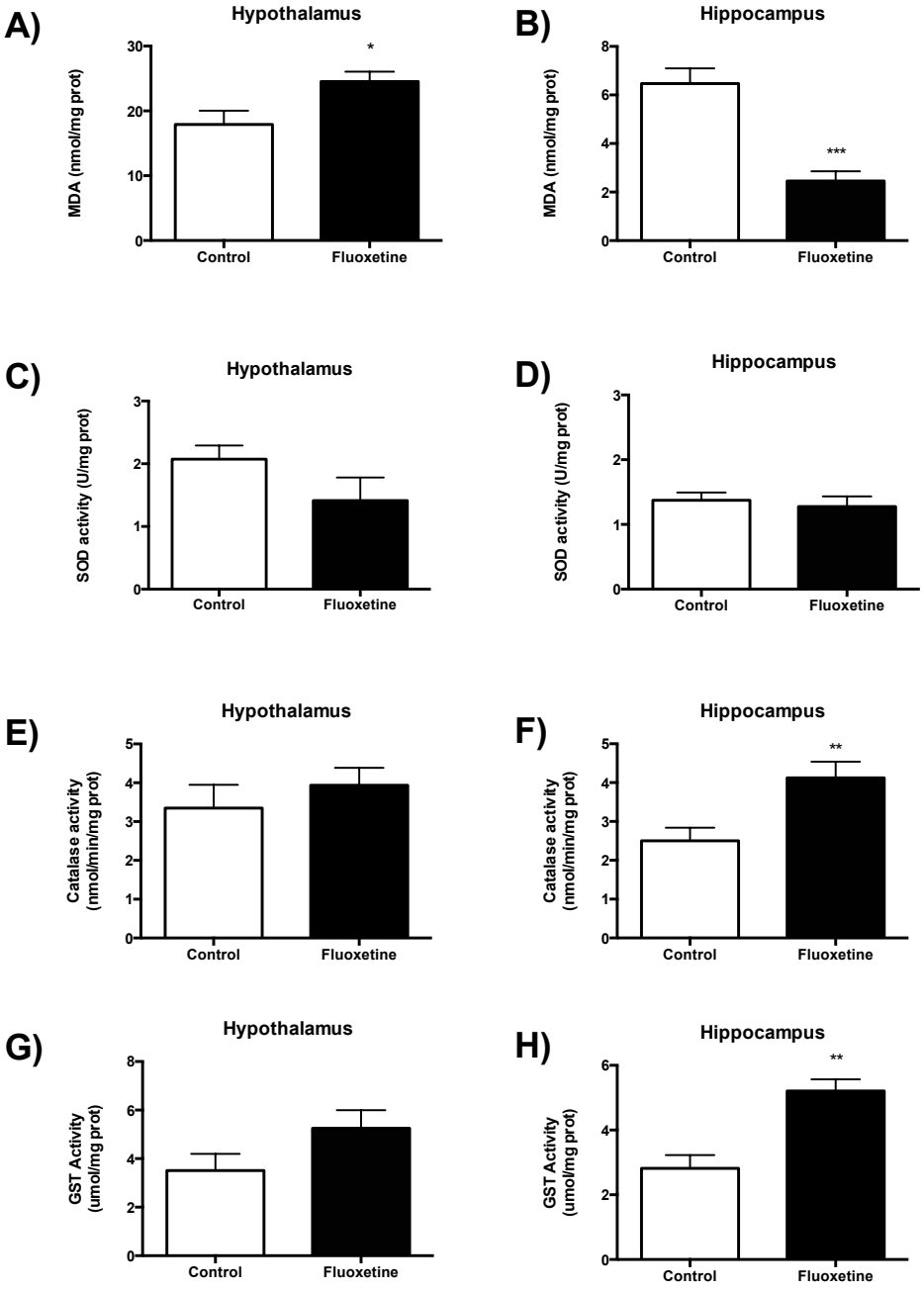

A)
Time spent on open arms (s)
150
100
50
0
*
Control
Fluoxetine
B)
Open arms entires (#)
15
10
5
0
*
Control
Fluoxetine
C)
Percentage of time on the open arms (%)
60
40
20
0
**
Control
Fluoxetine
D)
Percentage of entries on the open arms (%)
60
40
20
0
*
Control
Fluoxetine
E)
Time spent on close arms (s)
200
150
100
50
0
**
Control
Fluoxetine
F)
Percentage of entries on the close arms (%)
80
60
40
20
0
Control
Fluoxetine

FIGURE 5)


A)
Hypothalamus
MDA (nmol/mg prot)
30
20
10
0
*
Control
Fluoxetine
B)
Hippocampus
MDA (nmol/mg prot)
8
6
4
2
0
***
Control
Fluoxetine
C)
Hypothalamus
SOD activity (U/mg prot)
3
2
1
0
Control
Fluoxetine
D)
Hippocampus
SOD activity (U/mg prot)
3
2
1
0
Control
Fluoxetine
E)
Hypothalamus
Catalase activity (nmol/min/mg prot)
5
4
3
2
1
0
Control
Fluoxetine
F)
Hippocampus
Catalase activity (nmol/min/mg prot)
5
4
3
2
1
0
**
Control
Fluoxetine
G)
Hypothalamus
GST Activity (umol/mg prot)
8
6
4
2
0
Control
Fluoxetine
H)
Hippocampus
GST Activity (umol/mg prot)
6
4
2
0
**
Control
Fluoxetine

Apêndice B - ***"EFFECT OF FLUOXETINE TREATMENT ON MITOCHONDRIAL BIOENERGETIC IN CENTRAL AND PERIFERIC RAT TISSUES" foi submetido à revista Applied Physiology, Nutrition and Metabolism (Qualis A2 em Nutrição).***

Effect of fluoxetine treatment on mitochondrial bioenergetic in central and periferic rat tissues

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Running title: Chronic fluoxetine exposure modulates mitochondrial bioenergetics.

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Abstract

Mitochondria are the cellular organelles involved in many actions; currently several studies have been suggesting that the mitochondria could be a possible drug target to fight against metabolic diseases, althought studies exploring the role of mitochondria in central and peripheric tissues involved in the control of energy balance it's still little explored. With these, the aim of the present study was investigate the effects of chronic treatment with fluoxetine during the critical period of development, on the mitochondrial bioenergetics of the hypothalamus and skeletal muscle from male rats. Pups Wistar rats were injected (i.e subcutaneous) daily with fluoxetine (Fx group) or vehicle solution (Ct group) from 1 day of life until 21 days of life. At 60 days of age the mitochondrial bioenergetics was evaluated, in Fx-group showed an increase in oxygen consumption in different respiratory state, reduction in the production of reactive oxygen species and no changes in mitochondrial permeability transition pore opening and in oxidative stress in both tissues. Evaluating antioxidant enzymes we observed an increase in the GST activity only in the hypothalamus of Fx-group. Taken together our results suggest that chronic exposure to fluoxetine during a period of CNS development causes a positive modulation of the mitochondria function in the hypothalamus and skeletal muscle.

Keywords: central nervous system, energy balance, mitochondrial metabolism

1. INTRODUCTION

Mitochondria are the major organelles responsible for producing energy for cellular processes through the conversion and production of metabolites of adenosine-5'-triphosphate (ATP). In addition to the classic role, several studies already demonstrated that mitochondria are also involved in diverse functions, such as regulation of Reactive Oxygen Species (ROS) production, calcium balance, the redox signaling and cellular viability (i.e apoptosis) (Koopman et al. 2010).

Recent studies showed that mitochondrial dysfunction associated with increase in ROS production and oxidative damage is a major contributor to the energy unbalance in some diseases, such as obesity and diabetes (Aon et al. 2014; Hepple 2014; Jeong et al. 2014). The maintenance of energy balance requires several physiological and behavioral adjustments (Rios 2014) pathways that control the whole-body energy metabolism are integrated in the Central Nervous System (CNS) to produce adequate responses and maintain energy homeostasis (Williams et al. 2012; Jeong et al. 2014). The hypothalamus acts as determinate site in the control of energetic balance by processing and integrating central and peripheral information. Studies showed that under negative energy balance conditions, the activation of hypothalamic neurons induce a greater stimulation of peripheral tissues (eg skeletal muscle and adipose tissue), increasing metabolism and resulting in increases in fatty acids oxidation by mitochondrial β-oxidation process (Cha et al. 2005; Cha et al. 2006; Nasrallah et al. 2014).

Recent studies have shown that exposure to antidepressant drugs induce excessive production of ROS and affect the antioxidant defense system in different tissues (Novio et al. 2011; Moretti et al. 2012; De Long et al. 2014). The effect of antidepressant in redox status is stil controversy. (Zlatkovic et al. 2014) studing the effect of chronic treatment with fluoxetine and clozapine on liver injury had shown that fluoxetine appears to be less toxic than clozapine, beside fluoxetine increase oxidative stress biomarkers. In contrast, Aksu et al. (2014) testing the effect of fluoxetine as antioxidant, pre-treat with fluoxetine in ischemia-reperfusion (IR) kidney model. The authors reported that IR in kidney induce increases in oxidative stress, decrease of antioxidant defense and increases in pro-inflamatory interleukins, but pre-treatment with fluoxetine significantly restore redox balance and decreases inflammatory parameters (Aksu et al. 2014). Particularly in the brain, studies suggest that antidepressants or mood stabilizers may act through action on mitochondrial functions (Hroudova et al. 2012; Siwek et al. 2013; Hroudova et al. 2014). Additional studies suggest that serotonin exerts a protective effect by decreasing oxidative damage (Park et al. 2002; Avram et al. 2005; Moretti et al. 2012; Abdel Salam et al. 2013) however these studies were

performed after the period of CNS development. Fluoxetine (i.e Prozac or Serafem) is a drug widely used for treat depression in pregnant and lactating women, potentially exposing the fetus and/or baby during the pre and postnatal period of CNS development (Borue et al. 2007). The development of the CNS is particularly vulnerable to ROS due to low levels of antioxidants, high levels of polyunsaturated fatty acids and the great need for oxygen in neurobiochemical reactions (Halliwell 2006). Thus, it is very likely that early exposure to antidepressant drugs may affect the antioxidant defense system in brain regions, leading to permanent alterations in the tissues involved in energy balance in adult life. To our knowledge no previous studies was conducted to verify the effect of chronic treatment of fluoxetine during critical period of development on mitochondrial bioenergetics in key tissues associated to energy balance. Thus, the aim of the present study was to investigate the effects of chronic exposure to fluoxetine during the postnatal period of CNS development (i.e. lactation) on mitochondrial bioenergetics in hypothalamus and skeletal muscle in male rats.

2. MATERIAL AND METHODS

2.1 Animals

The animal use protocols for this study have been approved by the Ethics Committee for Animal Research at the Federal University of Pernambuco in accordance with the guidelines published in "Principles of Laboratory Animal Care" (NIH, Bethesda, USA) and Canadian Council on Animal Care (CCAC) guidelines (Ethical Protocol 23076.015276/2012-56). *Wistar* rats (Rattus norvegicus) were maintained at a room temperature of 23 ± 1 °C in a 12-h alternating light–dark cycle (light 6:00 a.m.–6:00 p.m.). At ninety-days old, rats were mated (1 females for 1 male). 6-Pregnant rats were then transferred to individual cages and at least four male offspring of each litter were used in the present study. The pharmacological treatment stated 24 hours after birth. In our experimental groups no significant differences in litter size has been observed. The dams received commercial chow *ad libitum*. After weaning, the rat pups receive the same diet as their mothers, also *ad libidum*.

2.2 Pharmacological treatment and experimental groups

All male neonates received a subcutaneous fluoxetine (10 mg/kg, dissolved in saline solution, 10 ml/kg, bw; Fx group) or vehicle (NaCl 0.9%, 10 ml/kg, bw; control-Ct group) injection once daily from the 1st to the 21st postnatal day (i.e., during the suckling period) (Silva et al. 2010; da Silva et al. 2014). To avoid a possible influence of the circadian rhythm

in these studies, injections were always administered between 7:00 a.m. and 8:00 a.m. (Sanchez et al. 2008; da Silva et al. 2014).

2.3 Mitochondria isolation

Hypothalamus and EDL mitochondria were prepared by homogenization followed by differential centrifugation. Male Wistar rats (at 60 days of age) were killed by decapitation, and tissues was immediately removed and homogenized in a mixture containing 225 mmol/L mannitol, 75 mmol/L sucrose, 1 mmol/L EGTA, 4 mmol/L HEPES (pH 7.2). The homogenate was centrifuged at 4000 rpm for 10 min in 4°C. The supernatant was carefully removed and centrifuged at 15000 rpm for 10 min in 4°C. The pellet was re-suspended in a buffer containing 250 mmol/L sucrose and 5 mmol/L HEPES (pH7.2). Mitochondrial protein concentration was determined spectrophotometrically according to Bradford (Bradford 1976) with BSA as the standard.

2.4 Mitochondrial oxygen consumption

Measurement of mitochondrial respiration was performed at 28°C in a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). The mitochondria were incubated in respiration buffer containing 120 mmol/L Potassium chloride (KCl), 5 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mmol/L Ethylene glycol tetracetic acid (EGTA), 5 mmol/L, Monopotassium phosphate (KH_2PO_4), (pH 7.2) and 0.2% BSA. Mitochondria were used at 0.5 mg protein/mL buffer. Mitochondrial respiration was measured with Complex I and II substrates, ADP (0.5 mmol/L), Oligomycin (1.2 mmol/L) and CCCP (1 mmol/L) (Lagranha et al. 2010; Nascimento et al. 2014).

2.5 Mitochondrial ROS production

Mitochondrial ROS production in isolated mitochondria was performed at 28°C using a fluorescent probe H_2DCFDA (5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester). This dye is non-fluorescent when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases it becomes fluorescent (Garcia-Ruiz et al. 1995). Briefly, mitochondrial suspensions (0.5 mg protein) were incubated in the presence of 1 μmol/L H_2DCFDA and fluorescence was monitored over 5 minutes of gentle shaking using a temperature-controlled spectrofluorimeter (OMEGA, USA) with excitation and emission wavelengths of 503 and 529 nm, respectively. ROS production

was evaluated using complex I substrate (10/2 mmol/L of glutamate/malate). Under these conditions, the linear increment in fluorescence for each reaction indicated the rate of ROS formation.

2.6 Mitochondrial pore opening

The capacity of the mitochondria to retain calcium has been used as a measure of the permeability transition pore and can be monitored by analyzing mitochondrial swelling (Maloyan et al. 2005) as a decrease in light absorbance at 540 nm. Calcium at 100 μmol/L was added to measure the capacity of the mitochondrial maintain pore closed (Blattner et al. 2001).

2.7 Oxidative stress evaluation in hypothalamus and EDL

Hypothalamus and EDL tissues was prepared from male Wistar rats (at 60 days of age) by homogenization followed by centrifugation. The tissues was homogenated in buffer with Tris 50 mM (pH 7.4), EDTA 1mM and NP40 0.1%, with PMSF 2 mM and orthovanodate 1mM.

2.7.1 Evaluation of malondialdehyde (MDA) production

A total of 0.3 mg/mL of homogenate was used to measure MDA production following reaction with thiobarbituric acid (TBA) at 100 °C according to the method of Draper (Draper et al. 1993; Nascimento et al. 2014). In the TBA test reaction, MDA, or MDA-like substances react to produce a pink pigment with a maximum absorption at 535 nm. The reaction was developed by the addition to the sample of 30% trichloroacetic acid and Tris-HCl (3 mmol/L) followed by thorough mixing and centrifugation at 2500g for 10 min. Supernatant was transferred to another tube and 0.8% TBA (v/v) was added before mixing and boiling for 30 min. After cooling, the absorbance of the organic phase was read at 535 nm in a spectrophotometer. Results were expressed as nmol per mg of protein.

2.7.2 Superoxide dismutase (SOD) assay

The determination of total superoxide dismutase enzyme activity (t-SOD) was performed according to the method of Misra and Fridovich (Misra et al. 1972). Supernatants (0.3 mg/mL) collected from homogenized tissues following centrifugation were incubated with 0.880 mL of sodium carbonate (0.05%, pH 10.2, 0.1 mmol/L EDTA) at 37 C. Thirty millimoles per liter of epinephrine (in 0.05% acetic acid) was added and SOD activity

measured the kinetics of inhibition of adrenaline auto-oxidation at 480 nm. Data are expressed at U/mg protein (Cardoso et al. 2012).

2.7.3 Catalase (CAT) assay

A total of 0.3 mg/mL of homogenates was used to measure CAT activity according to the method described by Aebi (Aebi 1984). The principle of the assay is based on the determination of the rate constant (k) of H_2O_2 decomposition, which in our conditions of temperature and pH was defined as 4.6×10^7. The rate constant of the enzyme was determined by measuring the change in absorbance (at 240 nm) per minute over a 4-min period, and the CAT activity was expressed as U/mg protein (da Silva et al. 2014).

2.7.4 Glutathione S-Transferase (GST) assay

A total of 0.3 mg/mL of homogenates was used to measure GST activity according to the method of Habig et al. by determination of absorbance at 340 nm after addition of 1 mmol/L of 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al. 1974). GST activity was calculated using the detection of the 2,4-dinitrophenyl-S-glutathione (DNP-SG). Based on its molecular absorbance, 1 enzymatic unit was defined as the amount of protein required to the form of 1 μmol/L DNP-SG per minute (Nascimento et al. 2014).

2.8 Statistical analysis

All of the results are expressed as the means ± SEM. A student's t-test was performed to assess significant differences between the two groups. The data were considered to be statistically significant when $p \leq 0.05$. All of the data were plotted, and the statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS

3.1 Fluoxetine treatment in hypothalamic mitochondria

To assess mitochondrial function in the hypothalamus after fluoxetine treatment first we examined the mitochondrial oxygen consumption and we observed that hypothalamus's mitochondria from Fx group has a higher basal respiration than Ct group (Ct: 5.1 ± 0.44 nmol O_2/min/mg prot, N=7; Fx: 10.8 ± 0.64 nmol O_2/min/mg prot, N=7; $p<0.01$); we also observed that after add an uncoupling protonophore (CCCP) the Fx group still showed a higher respiration rate than Ct group (Fx: 45.0 ± 2.5 nmol O_2/min/mg prot, N=7; Ct: 25.7 ± 2.9 nmol O_2/min/mg prot, N=7; $p<0.0001$) (Figure 1A). After we measure ROS production,

since several studies suggest that increase in mitochondrial oxygen consumption may decrease ROS production (Korshunov et al. 1997; Skulachev 1998; Turrens 2003; Sangle et al. 2010). As shown in figure 1B, mitochondria from FX group produced significantly less ROS than Ct group. Consistent with theses results we did not observe difference in mitochondrial pore opening neither oxidative stress levels in hypothalamus (Figure 1C and Figure 2)

3.2 Fluoxetine treatment in skeletal muscle mitochondria

After the observation of the fluoxetine treatment in central tissue we next evaluate mitochondrial function in the EDL muscle. Measuring mitochondrial oxygen consumption we observed that mitochondria from Fx group had a higher respiration in all states than Ct group (Basal: Ct-17.7 ± 1.33 nmol O_2/min/mg prot, N=7; Fx-27.6 ± 1.66 nmol O_2/min/mg prot, N=7; $p<0.001$; ADP-Stimulation: Ct-24.4 ± 1.8 nmol O_2/min/mg prot, N=7; Fx-38.2 ± 2.48 nmol O_2/min/mg prot, N=7; $p<0.001$; Resting: Ct-16.1 ± 1.0 nmol O_2/min/mg prot, N=7; Fx- 20.7 ± 1.58 nmol O_2/min/mg prot, N=7; $p<0.05$; CCCP: Ct-33.9 ± 2.2 nmol O_2/min/mg prot, N=7; Fx- 51.7 ± 2.05 nmol O_2/min/mg prot, N=7; $p<0.001$) (Figure 3A). After we measure ROS production and mitochondrial pore opening. As shown in figure 3B mitochondria from Fx group produced significantly less ROS than Ct group. Consistent with theses results we also did not observe difference in mitochondrial pore opening neither oxidative stress levels in EDL muscle (Figure 3C and Figure 4)

4. DISCUSSION

As far as we known there is no previous studies that examined the effect of chronically treatment with fluoxetine, during rat development, on mitochondrial bioenergetics in central and peripheric tissues. In this study, we have used fluoxetine treatment of male rats during nursing, a critical period in brain development, to assess possible relationships between mitochondrial bioenergetics and energy balance in tissues-related to the control of body weight. Our results show that increased levels of serotonin early in life in male rats decreases body weight (data not showed), increases mitochondrial respiration, decreases ROS production, and also no changes was observed in mitochondrial permeability transition pore opening and oxidative stress levels in the hypothalamus and skeletal muscle. These findings suggest that manipulation of the serotonergic system during critical periods of development can result in persistent changes in whole-body energy metabolism tissues-related.

In our experimental model we observed that fluoxetine induce increases in several mitochondrial respiration stage either in hypothalamus as in skeletal muscle. (Hroudova and Fisar 2012) studing *in vitro* the effect of fluoxetine among others antidepressant showed that fluoxetine at concentrations higher than 80 μmol/L and higher than 260 μmol/L inhibit complex I and complex II, respectively. In contrast, Agostinho et al (2011) have shown that acute treatment with fluoxetine in adult male rat induces increase in citrate synthase activity, the frist enzyme of Krebs cycle's, in striatum (Agostinho et al. 2011). Studies suggest that increase in Krebs cycle's products (i.e NADH and $FADH_2$) increases mitochondrial respiratory processes (Chance et al. 1956; Groen et al. 1982; Brown et al. 1990; Harris et al. 1991; Murphy 2001). In a different study, Abelaira et al (2011) showed that acute treatment in adult male rat with imipramine (serotonin reuptake inhibitor) increases the activity of citrate synthase and respiratory complex II activity in amygdala's area. In adition, in the same study the authors showed that chronic treatment with imipramine during 14 days (once a day) increases the activity of mitochondrial respiratory complex II and complex III in prefrontal cortex and hippocampus, beside the increase in mitochondrial respiratory complex III activity in amygdala (Abelaira et al. 2011). Combined previous data, with our data, suggests that fluoxetine *in vivo* increases mitochondrial oxygen consumption by increases in substrate oxidation.

In our results we observe in addition to increase in oxygen consumption a decrease in ROS production in both tissues. A variety of studies had shown that conditions, including oxidative phosphorylation or uncoupling, were increases oxygen consumption and electron transport chain (ETC) prevent mitochondrial ROS production in different tissues (Korshunov et al. 1997; Skulachev 1998; Sangle et al. 2010). Studies suggested that the mechanism involved in decreases of ROS production is related to the prevention of anion superoxide (O_2^-) formation by decreasing oxygen tension in the mitochondrial milieu (Skulachev 1998; Murphy 2009). Another possible mechanism involves the capacity of the ETC maintain NADH levels lower, which prevents ROS formation by mitochondrial matrix flavoenzymes (Starkov et al. 2004; Tretter et al. 2004). Another possibility is due to increased electron transport rates are often accompanied by lower mitochondrial membrane potential ($\Delta\Psi$), a condition that thermodynamically disfavors the reverse flow of electron from Complex II to Complex I, decreasing electron leak and O_2^- formation (Turrens 2003).

Previous studies suggested that inhibition of oxidative phosphorylation cause redirection of electrons from ETC, increasing ROS production, increasing oxidative stress,

and declination of energy production and MPTP opening (Kowaltowski et al. 2001; Wallace 2001; Halestrap et al. 2002). Mitochondrial permeability transition pore (MPTP) refers to an abrupt increase in the permeability of the inner mitochondrial membrane to low molecular weight molecules due to osmotic forces leading to the structural alteration with cell death mediated by necrosis or apoptosis (Gunter et al. 1990; Bernardi et al. 1996; Rasola et al. 2011). Our data related to mitochondrial respiration and ROS production suggested that MPTP in our model would not be induced since we observed the opposite effect (i.e increases in mitochondrial respiration and decreases in ROS production). Evaluating MPTP opening, we indeed did not observe any detrimental effect of fluoxetine treatment with or without 100 μM of calcium in hypothalamus or skeletal tissue, suggesting that fluoxetine does not induce impair in mitochondrial bioenergetics. In adition to non-effect in MPTP, we did not observe oxidative stress neither in hypothalamus or skeletal muscle, this effect endorses the results observed in mitochondrial respirations and lower levels of ROS released.

Taking our current data together with the available literature on mitochondrial bioenergetics, we suggest that fluoxetine induce body weight decrease by the increment in mitochondrial oxidative phosphorylation (i.e. Krebs cycle and ETC process). Such results suggest that early intervention with serotonin re-uptake inhibitors (like fluoxetine in this study) could contribute to decreases metabolic diseases, perhaps by improving mitochondrial function with reductions in oxidative stress.

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LEGENDS:

Figure 1 - Effect of chronic treatment with fluoxetine on oxygen consumption (A), production of reactive oxygen species (B) and mitochondrial pore opening (C) in the hypothalamus of male rats with 60 days of life. Was used succinate (10 mM) as mitochondrial substrate and 10 mM CaCl2. The pups received daily fluoxetine (Fx = 10 mg / kg bw, sc, n = 7) or vehicle (0.9% NaCl, C = 10 ml / kg bw, sc, n = 7) from the 1st to 21th day of life. Data are presented as mean ± SEM.

Figure 2 - Effect of chronic exposure to fluoxetine on oxidative balance (A-MDA, B-SOD, C-Catalase, D-GST) in the hypothalamus of male rats with 60 days of life. The pups received daily fluoxetine (Fx = 10 mg / kg bw, sc, n = 7) or vehicle (0.9% NaCl C = 10 ml / kg bw, sc, n = 7) from the 1st to 21th day of life. Data are presented as mean ± SEM.

Figure 3 - Effect of chronic treatment with fluoxetine on oxygen consumption (A), production of reactive oxygen species (B) and mitochondrial pore opening (C) in the EDL muscle of male rats with 60 days of life. Was used succinate (10 mM) as mitochondrial substrate and 10 mM CaCl2. The pups received daily fluoxetine (Fx = 10 mg / kg bw, sc, n = 7) or vehicle (0.9% NaCl C = 10 ml / kg bw, sc, n = 7) from the 1st to 21th day of life. Data are presented as mean ± SEM.

Figure 4 - Effect of chronic exposure to fluoxetine on oxidative balance (MDA A-, B-SOD, Catalase C-, D-GST) in the EDL muscle of male rats with 60 days of life. The pups received daily fluoxetine (Fx = 10 mg / kg bw, sc, n = 7) or vehicle (0.9% NaCl C = 10 ml / kg bw, sc, n = 7) from the 1st to 21th day of life. Data are presented as mean ± SEM.

FIGURES

Figure 1

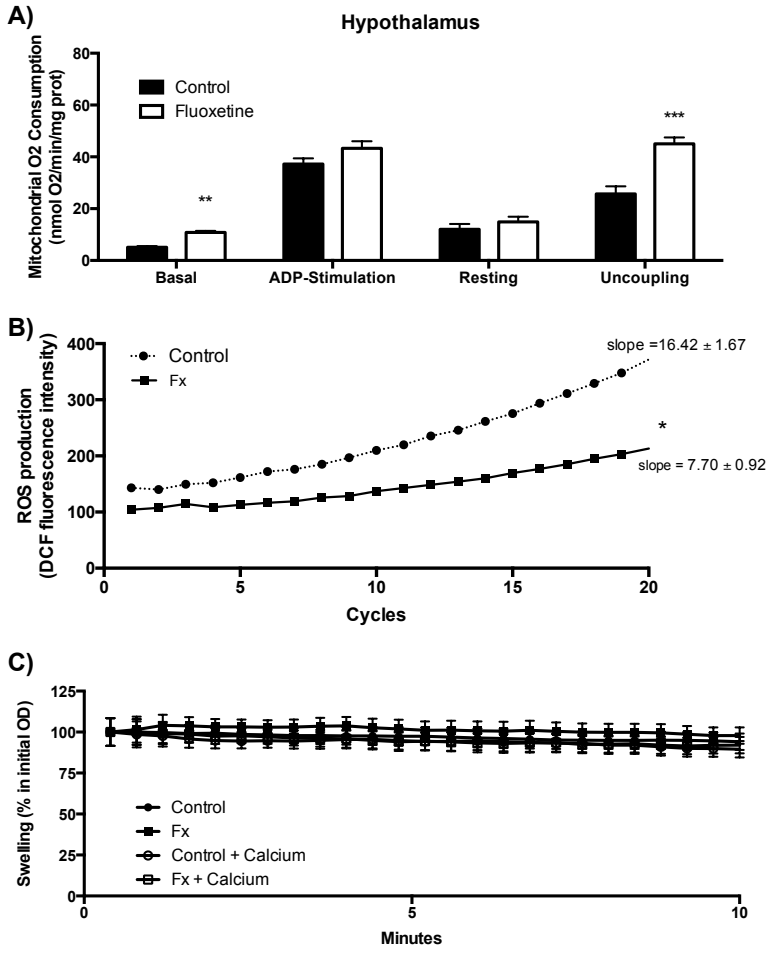


Figure 2

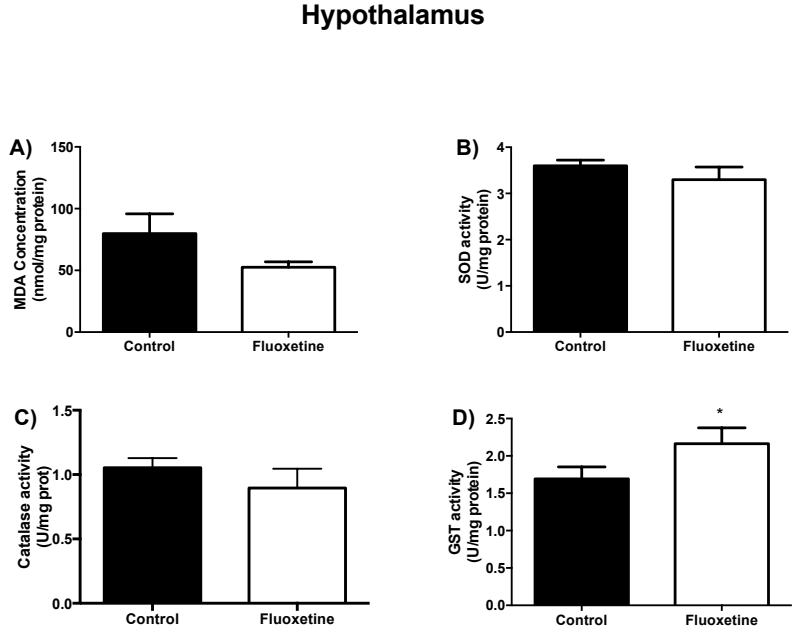


Figure 3

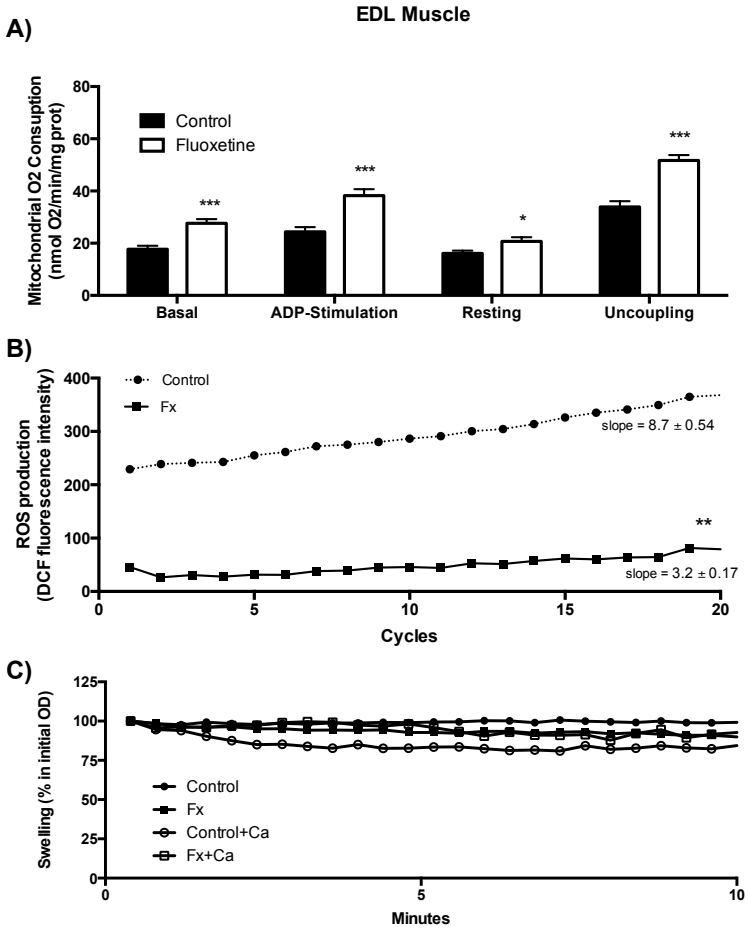
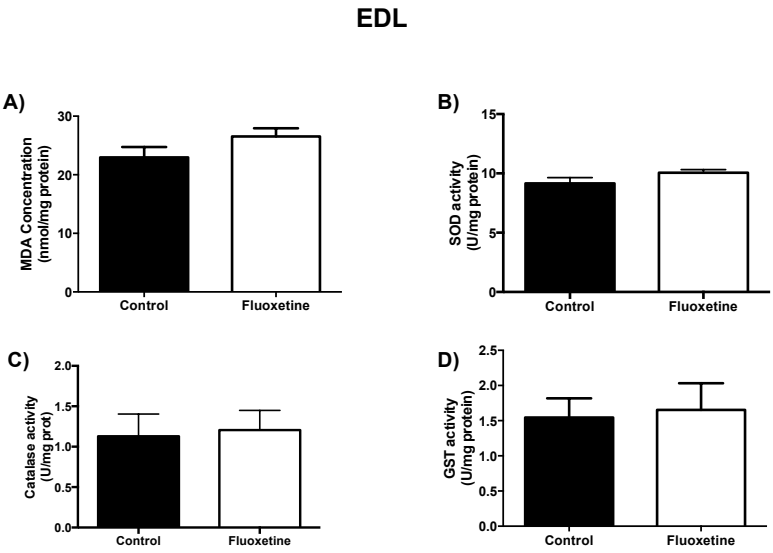


Figure 4



REFERENCES:

Abdel Salam OM, Mohammed NA, Sleem AA, andFarrag AR. 2013. The effect of antidepressant drugs on thioacetamide-induced oxidative stress. Eur Rev Med Pharmacol Sci **17**: 735-44.

Abelaira HM, Reus GZ, Ribeiro KF, Zappellini G, Ferreira GK, Gomes LM, Carvalho-Silva M, Luciano TF, Marques SO, Streck EL, Souza CT, andQuevedo J. 2011. Effects of acute and chronic treatment elicited by lamotrigine on behavior, energy metabolism, neurotrophins and signaling cascades in rats. Neurochem Int **59**: 1163-74.

Aebi H. 1984. Catalase in vitro. Methods Enzymol **105**: 121-6.

Agostinho FR, Reus GZ, Stringari RB, Ribeiro KF, Ferraro AK, Benedet J, Rochi N, Scaini G, Streck EL, andQuevedo J. 2011. Treatment with olanzapine, fluoxetine and olanzapine/fluoxetine alters citrate synthase activity in rat brain. Neurosci Lett **487**: 278-81.

Aksu U, Guner I, Yaman OM, Erman H, Uzun D, Sengezer-Inceli M, Sahin A, Yelmen N, Gelisgen R, Uzun H, andSahin G. 2014. Fluoxetine ameliorates imbalance of redox homeostasis and inflammation in an acute kidney injury model. J Physiol Biochem.

Aon MA, Bhatt N, andCortassa SC. 2014. Mitochondrial and cellular mechanisms for managing lipid excess. Front Physiol **5**: 282.

Avram AS, Avram MM, andJames WD. 2005. Subcutaneous fat in normal and diseased states: 2. Anatomy and physiology of white and brown adipose tissue. J Am Acad Dermatol **53**: 671-83.

Bernardi P and Petronilli V. 1996. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. J Bioenerg Biomembr **28**: 131-8.

Blattner JR, He L, andLemasters JJ. 2001. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. Anal Biochem **295**: 220-6.

Borue X, Chen J, andCondron BG. 2007. Developmental effects of SSRIs: lessons learned from animal studies. Int J Dev Neurosci **25**: 341-7.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**: 248-54.

Brown GC, Lakin-Thomas PL, andBrand MD. 1990. Control of respiration and oxidative phosphorylation in isolated rat liver cells. Eur J Biochem **192**: 355-62.

Cardoso HD, Passos PP, Lagranha CJ, Ferraz AC, Santos Junior EF, Oliveira RS, Oliveira PE, Santos Rde C, Santana DF, Borba JM, Rocha-de-Melo AP, Guedes RC, Navarro DM, Santos GK, Borner R, Picanco-Diniz CW, Beltrao EI, Silva JF, Rodrigues MC, andAndrade da Costa BL. 2012. Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids. Front Hum Neurosci **6**: 249.

Cha SH, Hu Z, Chohnan S, andLane MD. 2005. Inhibition of hypothalamic fatty acid synthase triggers rapid activation of fatty acid oxidation in skeletal muscle. Proc Natl Acad Sci U S A **102**: 14557-62.

Cha SH, Rodgers JT, Puigserver P, Chohnan S, andLane MD. 2006. Hypothalamic malonyl-CoA triggers mitochondrial biogenesis and oxidative gene expression in skeletal muscle: Role of PGC-1alpha. Proc Natl Acad Sci U S A **103**: 15410-5.

Chance B and Williams GR. 1956. The respiratory chain and oxidative phosphorylation. Adv Enzymol Relat Subj Biochem **17**: 65-134.

da Silva AI, Galindo LCM, Nascimento L, Freitas CM, Manhaes-de-Castro R, Lagranha CJ, andSouza SL. 2014. Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life. Can J Physiol Pharmacol.
De Long NE, Hyslop JR, Raha S, Hardy DB, andHolloway AC. 2014. Fluoxetine-induced pancreatic beta cell dysfunction: New insight into the benefits of folic acid in the treatment of depression. J Affect Disord **166**: 6-13.
Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, andHadley M. 1993. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radic Biol Med **15**: 353-63.
Garcia-Ruiz C, Colell A, Morales A, Kaplowitz N, andFernandez-Checa JC. 1995. Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-kappa B: studies with isolated mitochondria and rat hepatocytes. Mol Pharmacol **48**: 825-34.
Groen AK, Wanders RJ, Westerhoff HV, van der Meer R, andTager JM. 1982. Quantification of the contribution of various steps to the control of mitochondrial respiration. J Biol Chem **257**: 2754-7.
Gunter TE and Pfeiffer DR. 1990. Mechanisms by which mitochondria transport calcium. Am J Physiol **258**: C755-86.
Habig WH, Pabst MJ, Fleischner G, Gatmaitan Z, Arias IM, andJakoby WB. 1974. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proc Natl Acad Sci U S A **71**: 3879-82.
Halestrap AP, McStay GP, andClarke SJ. 2002. The permeability transition pore complex: another view. Biochimie **84**: 153-66.
Halliwell B. 2006. Oxidative stress and neurodegeneration: where are we now? J Neurochem **97**: 1634-58.
Harris DA and Das AM. 1991. Control of mitochondrial ATP synthesis in the heart. Biochem J **280 (Pt 3)**: 561-73.
Hepple RT. 2014. Mitochondrial involvement and impact in aging skeletal muscle. Front Aging Neurosci **6**: 211.
Hroudova J and Fisar Z. 2012. In vitro inhibition of mitochondrial respiratory rate by antidepressants. Toxicol Lett **213**: 345-52.
Hroudova J, Singh N, andFisar Z. 2014. Mitochondrial dysfunctions in neurodegenerative diseases: relevance to Alzheimer's disease. Biomed Res Int **2014**: 175062.
Jeong JK, Kim JG, andLee BJ. 2014. Participation of the central melanocortin system in metabolic regulation and energy homeostasis. Cell Mol Life Sci **71**: 3799-809.
Koopman WJ, Nijtmans LG, Dieteren CE, Roestenberg P, Valsecchi F, Smeitink JA, andWillems PH. 2010. Mammalian mitochondrial complex I: biogenesis, regulation, and reactive oxygen species generation. Antioxid Redox Signal **12**: 1431-70.
Korshunov SS, Skulachev VP, andStarkov AA. 1997. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett **416**: 15-8.
Kowaltowski AJ, Castilho RF, andVercesi AE. 2001. Mitochondrial permeability transition and oxidative stress. FEBS Lett **495**: 12-5.
Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, andMurphy E. 2010. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. Circ Res **106**: 1681-91.

Maloyan A, Sanbe A, Osinska H, Westfall M, Robinson D, Imahashi K, Murphy E, andRobbins J. 2005. Mitochondrial dysfunction and apoptosis underlie the pathogenic process in alpha-B-crystallin desmin-related cardiomyopathy. Circulation **112**: 3451-61.
Misra HP and Fridovich I. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem **247**: 3170-5.
Moretti M, Colla A, de Oliveira Balen G, Dos Santos DB, Budni J, de Freitas AE, Farina M, andSevero Rodrigues AL. 2012. Ascorbic acid treatment, similarly to fluoxetine, reverses depressive-like behavior and brain oxidative damage induced by chronic unpredictable stress. J Psychiatr Res **46**: 331-40.
Murphy MP. 2001. How understanding the control of energy metabolism can help investigation of mitochondrial dysfunction, regulation and pharmacology. Biochim Biophys Acta **1504**: 1-11.
Murphy MP. 2009. How mitochondria produce reactive oxygen species. Biochem J **417**: 1-13.
Nascimento L, Freitas CM, Silva-Filho R, Leite AC, SILVA AB, da Silva AI, Ferreira DJ, Pedroza AA, Maia MBS, Fernandes MP, andLagranha C. 2014. The effect of maternal low-protein diet on the heart of adult-offspring: Role of mitochondria and oxidative stress. Applied Physiology, Nutrition, and Metabolism.
Nasrallah CM and Horvath TL. 2014. Mitochondrial dynamics in the central regulation of metabolism. Nat Rev Endocrinol **10**: 650-658.
Novio S, Nunez MJ, Amigo G, andFreire-Garabal M. 2011. Effects of fluoxetine on the oxidative status of peripheral blood leucocytes of restraint-stressed mice. Basic Clin Pharmacol Toxicol **109**: 365-71.
Park JW, Youn YC, Kwon OS, Jang YY, Han ES, andLee CS. 2002. Protective effect of serotonin on 6-hydroxydopamine- and dopamine-induced oxidative damage of brain mitochondria and synaptosomes and PC12 cells. Neurochem Int **40**: 223-33.
Rasola A and Bernardi P. 2011. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium **50**: 222-33.
Rios M. 2014. Neurotrophins and the regulation of energy balance and body weight. Handb Exp Pharmacol **220**: 283-307.
Sanchez S, Sanchez C, Paredes SD, Cubero J, Rodriguez AB, andBarriga C. 2008. Circadian variations of serotonin in plasma and different brain regions of rats. Mol Cell Biochem **317**: 105-11.
Sangle GV, Chowdhury SK, Xie X, Stelmack GL, Halayko AJ, andShen GX. 2010. Impairment of mitochondrial respiratory chain activity in aortic endothelial cells induced by glycated low-density lipoprotein. Free Radic Biol Med **48**: 781-90.
Silva CM, Goncalves L, Manhaes-de-Castro R, andNogueira MI. 2010. Postnatal fluoxetine treatment affects the development of serotonergic neurons in rats. Neurosci Lett **483**: 179-83.
Siwek M, Sowa-Kucma M, Dudek D, Styczen K, Szewczyk B, Kotarska K, Misztakk P, Pilc A, Wolak M, andNowak G. 2013. Oxidative stress markers in affective disorders. Pharmacol Rep **65**: 1558-71.
Skulachev VP. 1998. Uncoupling: new approaches to an old problem of bioenergetics. Biochim Biophys Acta **1363**: 100-24.
Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, andBeal MF. 2004. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. J Neurosci **24**: 7779-88.
Tretter L and Adam-Vizi V. 2004. Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase. J Neurosci **24**: 7771-8.

Turrens JF. 2003. Mitochondrial formation of reactive oxygen species. J Physiol **552**: 335-44.
Wallace DC. 2001. A mitochondrial paradigm for degenerative diseases and ageing. Novartis Found Symp **235**: 247-63; discussion 263-6.
Williams KW and Elmquist JK. 2012. From neuroanatomy to behavior: central integration of peripheral signals regulating feeding behavior. Nat Neurosci **15**: 1350-5.
Zlatkovic J, Todorovic N, Tomanovic N, Boskovic M, Djordjevic S, Lazarevic-Pasti T, Bernardi RE, Djurdjevic A, andFilipovic D. 2014. Chronic administration of fluoxetine or clozapine induces oxidative stress in rat liver: a histopathological study. Eur J Pharm Sci **59**: 20-30.

Apêndice C- ***"NEONATAL MANIPULATION OF THE SEROTONIN ALTERS ENERGY BALANCE: PARTICIPATION OF MITOCHONDRIA AND UCP IN BROWN FAT TISSUE", foi submetido à revista Biochimica et Biophysica Acta- Bioenergetics (Qualis A1 em Nutrição).***

Neonatal manipulation of the serotonin alters energy balance: Participation of mitochondria and UCP in brown fat tissue

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Running title: Chronic fluoxetine exposure during brain development modulates UCP.

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ABSTRACT

BACKGROUND: The serotonergic system plays a crucial role in the development and homeostasis of the eating behavior and energy balance regulation. Energy balance is mediated by food intake and caloric expenditure. Thus, the present study investigated the mechanisms that might be associated with fluoxetine treatment-induced weight reduction. **METHODS**: Wistar male rat pups were injected daily with subcutaneous fluoxetine (Fx-group) or vehicle solution (Ct-group) until 21 days of age (days). Several analyses were conducted to verify the involvement of mitochondria in weight reduction. **RESULTS**: We found that body weight in the Fx-group was lower compared to control. In association to lower fat mass in the Fx-group (25%). Neither neonatal caloric intake nor food intake reveals significant differences. Evaluating caloric expenditure (locomotor activity and thermogenesis), we did not observe differences in locomotor activity. However, we observed that the Fx-group displayed 30% less heat loss compared with the Ct-group. Since brown adipose tissue (BAT) is specialized for heat production and the rate of heat production is related to mitochondrial function, we found that Fx-treatment increases respiration by 36%, although after addition of GDP respiration returned to Ct-levels. Examining ROS production and oxidative stress the Fx-group produced less ROS and no difference was observed in oxidative stress. Evaluating UCP expression we found that Fx-treatment increase UCP expression by 23%. **CONCLUSIONS**: Taken together, our results suggest that serotonin modulates energy balance possible due to mitochondrial respiration and UCP activation. **GENERAL SIGNIFICANCE:** Body weight modulated by fluoxetine treatment involves mitochondrial activity and Uncoupling proteins (UCP) in BAT.

Keywords: fluoxetine, developmental period, body weight, energy balance, mitochondria

1. INTRODUCTION

The serotonin (5-HT) that is produced in the brain contributes substantially to food intake and energy expenditure regulation [1-3]. During the perinatal period, serotonin has an important effect on target cells and organs to modulate development [4-7]. It is suggested that early exposure to selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine can impact the pattern of brain development [8]. A study from Silva et al. (2010) using fluoxetine during lactation demonstrated morphological changes in serotonergic neurons in addition to a gradual reduction in body weight during lactation and post-weaning [9]. SSRIs are a class of antidepressants that are often prescribed to pregnant and lactating women with varying degrees of depression, thus exposing fetuses and infants to the drug during brain development [10].

Maintaining energy balance requires several physiological and behavioral adjustments to obtain energy, especially controlling metabolism and storage [11]. Energy imbalance is the main cause of the global epidemics such as obesity and diabetes mellitus [12]. Pathways that control body nutritional status and energy levels are integrated into the Central Nervous System (CNS) to produce appropriate responses in peripheral tissues (i.e brown adipose tissue or skeletal muscle) and maintain energy homeostasis [13]. The cerebral serotoninergic system, especially hypothalamic nucleus, contributes to the regulation of energy expenditure [2, 3]. Studies show that electrical stimulation of hypothalamic nucleus leads to activation of brown adipose tissue and increase body temperature [14]. These authors suggested that this signal is likely mediated by serotonin [14]. Bross and Hoffer (1995) demonstrated that fluoxetine administration increased energy expenditure, which was associated with increased basal body temperature in humans [15]. A study using sibutramine showed activation of brown adipose tissue indirectly through activation of sympathetic system [16]; other studies using fenfluramine (stimulates serotonin release) showed activation of brown adipose tissue with a decrease in metabolic efficiency (uncouple electron transport from ATP synthesis and with generate heat in brown adipose tissue mitochondria) [17-20].

Since mechanistic control of energy balance needs the participation of the central nervous system, which is vulnerable to environmental stimuli during the critical developmental period [21], it is very likely that serotonin system manipulation by fluoxetine treatment during development is crucial to developing specific and permanent adaptation, which could reflect in brown adipose tissue activation. The mechanisms involved in these early fluoxetine treatment–induced adaptations have not been fully elucidated. No experimental studies have been conducted to investigate the role of developmental serotonin

on energy balance and the participation of brown adipose tissue mitochondria. Thus, to investigate whether serotonergic system manipulation during development would affect energy balance, we conducted several experiments to investigate what was responsible for the gain and loss of weight (energy).

2. MATERIALS AND METHODS

2.1 Animals

The animal use protocols for this study have been approved by the Ethics Committee for Animal Research at the Federal University of Pernambuco in accordance with the guidelines published in "Principles of Laboratory Animal Care" (NIH, Bethesda, USA) and Canadian Council on Animal Care (CCAC) guidelines (Ethical Protocol 23076.015276/2012-56). Wistar rats (*Rattus norvegicus*) were maintained at a room temperature of 23 ± 1 °C in a 12-h alternating light–dark cycle (light 6:00 a.m.–6:00 p.m.). At ninety-days old, rats were mated (1 females for 1 male). 6-Pregnant rats were then transferred to individual cages and at least four male offspring of each litter were used in the present study. The pharmacological treatment stated 24 hours after birth. In our experimental groups no significant differences in litter size has been observed. The dams received commercial chow *ad libitum*. After weaning, the rat pups receive the same diet as their mothers, also *ad libidum*.

2.2 Pharmacological treatment and experimental groups

All male neonates received a subcutaneous fluoxetine (10 mg/kg, dissolved in saline solution, 10 ml/kg, bw; Fx group) or vehicle (NaCl 0.9%, 10 ml/kg, bw; control-Ct group) injection once daily from the 1st to the 21st postnatal day (i.e., during the suckling period) [9, 22]. To avoid a possible influence of the circadian rhythm in these studies, injections were always administered between 7:00 a.m. and 8:00 a.m. [22, 23].

2.3 Body weight measurement

Body weights (in grams) were measured from 1st to the 21st postnatal day (weaning) and again at 40 and 60 days after birth using a digital balance (Marte, model S-100 with a 0.001 g sensitivity) [22, 24].

2.4 Food intake measurement

2.4.1 Neonatal food intake

Individual pup food consumption in milligrams (mg) was obtained on lactation day 7, 14 and 21. To stimulate food intake, animals were separated from their mother for 3 hours. During this period, pups aged 7 and 14 days stayed together in a plastic box in a 33°C incubator. Consumption was measured by weight gain one hour after return to the mother box [25]. To ensure greater food intake quantitation reliability, the stimulation was performed with soft object genital cups to promote urine and feces excretion [26].

2.4.2 24-hour food intake evaluation

Food intake (in grams) was evaluated by weighing commercial chow (Labina®) consumption for 24 hours at 40 days of age.

2.5 Brown and White adipose tissue quantification

Intrascapular brown adipose tissue, mesenteric and epididymal white adipose tissue were excised, rinsed and weighed (in grams) using a digital balance (Marte, model S-100 with a 0.001 g sensitivity) at postnatal day 60.

2.6 Free locomotor activity measurement

At 40 days of age, the pups were individually recorded for 24 hours using an infrared camera (1/3480 chip sony line), and the captured images were stored in a computer system for voluntary activity analysis. Free locomotor activity including combined exploratory behaviors (i.e., holding the cage, nest building and grooming behavior) was recorded [27].

2.7 Rectal body temperature measurement after thermal stimulation

At 40 days of age, the animal rectal body temperature (in °C) was assessed. The record was initially performed at room temperature, and the animals were then placed in a container with air vents and subjected to freezing ambient at -15°C, and rectal temperature was measured every 30 minutes for 90 minutes [28].

2.8 Mitochondria isolation

Brown Adipose Tissue (BAT) mitochondria were prepared by homogenization followed by differential centrifugation. Male Wistar rats (at 60 days of age) were killed by decapitation, and BAT was immediately removed from interscapular deposits and

homogenized in a mixture containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 4 mM HEPES (pH 7.2). The homogenate was centrifuged at 4000 rpm for 10 min in 4°C. The supernatant was carefully removed and centrifuged at 15000 rpm for 10 min in 4°C. The pellet was re-suspended in a buffer containing 250mM sucrose and 5mM HEPES (pH7.2). Mitochondrial protein concentration was determined spectrophotometrically according to Bradford with BSA as the standard.

2.9 Mitochondrial oxygen consumption

Measurement of mitochondrial respiration from BAT was performed at 28°C in a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). The mitochondria were incubated in respiration buffer containing 120 mmol/L Potassium chloride (KCl), 5 mmol/L 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mmol/L Ethylene glycol tetracetic acid (EGTA), 5 mmol/L, Monopotassium phosphate (KH2PO4), and 0.2% BSA. Mitochondria were used at 0.5 mg protein/mL buffer. Mitochondrial respiration was measured with Complex II (succinate 5mM) substrates, GDP (UCP inhibitor - 1mM) and CCCP (1 mM) [29, 30].

2.10 Mitochondrial reactive oxygen species (ROS) production

Mitochondrial ROS production in isolated mitochondria was performed at 28°C using a fluorescent probe DCFDA (5- (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester). This dye is non-fluorescent when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases it becomes fluorescent [31]. Briefly, mitochondrial suspensions (0.5 mg protein) were incubated in the presence of 1 µM DCFDA and fluorescence was monitored over 5 minutes of gentle shaking using a temperature-controlled spectrofluorimeter (OMEGA, USA) with excitation and emission wavelengths of 503 and 529 nm, respectively. Under these conditions, the linear increment in fluorescence for each reaction indicated the rate of ROS formation.

2.11 Oxidative stress evaluation in BAT

Brown Adipose Tissue (BAT) was prepared from male Wistar rats (at 60 days of age) by homogenization followed by centrifugation.

2.11.1 Evaluation of malondialdehyde (MDA) production

A total of 0.3 mg/mL of BAT homogenate was used to measure MDA production following reaction with thiobarbituric acid (TBA) at 100 °C according to the method of

Draper [30, 32]. In the TBA test reaction, MDA, or MDA-like substances react to produce a pink pigment with a maximum absorption at 535 nm. The reaction was developed by the addition to the sample of 30% trichloroacetic acid and Tris-HCl (3 mmol/L) followed by thorough mixing and centrifugation at 2500g for 10 min. Supernatant was transferred to another tube and 0.8% TBA (v/v) was added before mixing and boiling for 30 min. After cooling, the absorbance of the organic phase was read at 535 nm in a spectrophotometer. Results were expressed as nmol per mg of protein.

2.11.2 Superoxide dismutase (SOD) assay

The determination of total superoxide dismutase enzyme activity (t-SOD) was performed according to the method of Misra and Fridovich [33]. Supernatants (0.3 mg/mL) collected from homogenized BAT following centrifugation were incubated with 0.880 mL of sodium carbonate (0.05%, pH 10.2, 0.1 mmol/L EDTA) at 37^{o} C. Thirty millimoles per liter of epinephrine (in 0.05% acetic acid) was added and SOD activity measured the kinetics of inhibition of adrenaline auto-oxidation at 480 nm. Data are expressed at U/mg protein [34].

2.11.3 Catalase (CAT) assay

A total of 0.3 mg/mL BAT homogenate was used to measure CAT activity according to the method described by Aebi [35]. The principle of the assay is based on the determination of the rate constant (k) of H_2O_2 decomposition, which in our conditions of temperature and pH was defined as 4.6 x 10^7. The rate constant of the enzyme was determined by measuring the change in absorbance (at 240 nm) per minute over a 4-min period, and the CAT activity was expressed as U/mg protein [34].

2.11.4 Glutathione S-Transferase (GST) assay

A total of 0.3 mg/mL BAT homogenate was used to measure GST activity according to the method of Habig et al. by determination of absorbance at 340 nm after addition of 1 mmol/L of 1-chloro-2,4-dinitrobenzene [36]. GST activity was calculated using the detection of the 2,4-dinitrophenyl-S-glutathione (DNP-SG). Based on its molecular absorbance, 1 enzymatic unit was defined as the amount of protein required to the form of 1 mmol/L DNP-SG [30].

2.12 Western blot for UCPs expression

After mitochondrial isolation, aliquots of BAT mitochondria were used for the measurement of total protein content as described by Bradford. Equal amounts of proteins were separated using 14% SDS-gel polyacrylamide electrophoresis. Western blotting was carried out following the method described by Towbin et al. The proteins of the gel were transferred to a nitrocellulose membrane at 100V for 1 h. Non-specific binding was blocked by incubating the membranes with 5% defatted milk in basal solution (10mM Trizma, pH 7.5; 150mM NaCl; 0.05% Tween 20) overnight at 4 °C. Membranes were washed in basal solution three times for 5 min each and then incubated with anti-UCP1 antibodies (SCBT: SC-6528) in basal solution containing 3% defatted milk, at 4°C, overnight. Membranes were washed again (three times for 5min each) and incubated with anti-IgG antibody linked to horseradish peroxidase in basal solution containing 1% defatted milk, at 25°C, for 4 h. Following another washing, membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (ECL Western Blotting System) for 5 min and immediately exposed to X-ray film. Films were then processed in a conventional manner. Gel transfer efficiency and equal load was verified using reversible Ponceau staining [29]. Band intensities of the Western blotting experiments were analyzed and quantified by optical densitometry using the Image J software (NIH, Maryland, USA) [37, 38].

2.13 Statistical analysis

All of the results are expressed as the means ± SEM. A student's *t*-test was performed to assess significant differences between the two groups. Two-way ANOVA with multiple comparisons was used for repeated analyses. The data were considered to be statistically significant when $p \leq 0.05$. All of the data were plotted, and the statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS

3.1 Body weight evaluation

We measured body weight daily, and we observed that body weight was significantly lower in the treated group than the control group beginning in second week of life (Figure 1; 14 days after birth, Ct: 32.30 ± 0.5673 grams, n: 35, Fx: 25.10 ± 0.2900 grams, n: 35; 21st days, Ct: 50.70 ± 0.9768 grams, n: 35, Fx: 41.41 ± 0.7332 grams, n: 35,). Furthermore, body weights of the treated group remained lower than the control rats at 40th and 60th days (19 and 39 days after last drug application, respectively) (40th days: Ct: 131.8 ± 3.5 grams, n: 11, Fx: 115.0 ± 2.5 grams, n:16; 60th days: Ct: 239.10 ± 10.83 grams, n: 13, Fx: 206.1 ± 9.94 grams, n: 11).

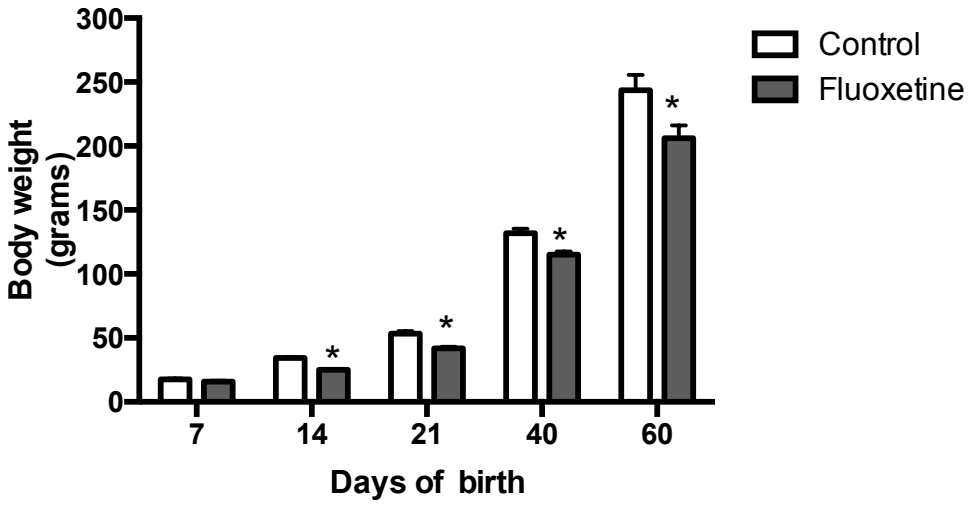

Figure 1 - Effect of neonatal fluoxetine treatment on body weight in male rat pups that were treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc) during the suckling period. Body weight on the 7th, 14th, 21st, 40th and 60th day of postnatal life is demonstrated. Body weight data are presented as the mean ± SEM (* $p < 0.001$).

3.2 Food intake

3.2.1 Neonatal Food Consumption

No differences were observed in food consumption between groups on the 7th days (Ct: 2.04 ± 0.19 grams, n: 16; Fx = 1.86 ± 0.17 grams, n:21); 14th days (Ct: 2.47 ± 0.31 grams, n:16; Fx: 2.24 ± 0.22 grams, n:21) and 21st days (Ct: 3.7 ± 0.18 grams, n:16; Fx: 3.05 ± 0.29 grams, n:21) (Figure 2).

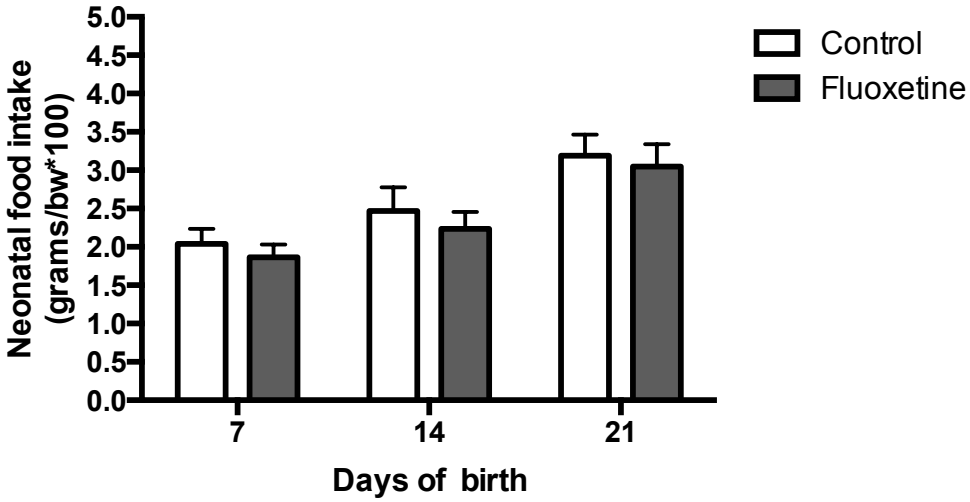

Figure 2 - Effect of neonatal food intake after deprivation in young male rats treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc, n = 21) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc, n = 16) during suckling period. Food intake on the 7th, 14th and 21th day of life was reported. Data on food intake are presented as the mean ± SEM.

3.2.2 24-hour food intake

To understand the possible mechanisms behind the decrease in body weight, we evaluated food intake during a 24-hour period in rats at 40 days. Interestingly, 24 h food intake was not altered by the drug, suggesting that the weight differences between the groups were not related to food intake (Ct: 19.5 ± 1.53 grams, n:16; Fx: 16.84 ± 1.139 grams, n:21) (Figure 3).

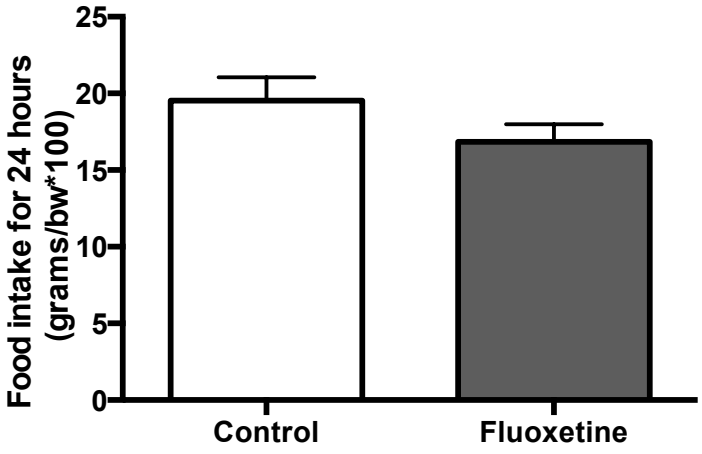

Figure 3 - Effect of neonatal fluoxetine treatment on food intake for 24 hours in pups of male rats treated with fluoxetine (fluoxetine = 10 mg/kg bw, sc, n = 8) or saline (control = 0.9%

NaCl 10 ml/kg ch, sc, n = 8) during suckling period. Food intake (g / bw * 100) on the 40th day of life was measured. The data are presented as the mean ± SEM.

3.3 White and Brown Adipose tissue weight

Because the results revealed no difference in food intake even though there was a difference in body weight, we quantified the adipose tissue weight. We observed that rats from the Fx group had significantly reduced white adipose tissue (Fx: 0.97 ± 0.093 grams, n: 11; $p<0.01$) compared with the control group (Ct: 1.72 ± 0.129 grams, n: 11) without changes in the amount of brown adipose tissue (Fx: 0.085 ± 0.005 grams, n: 9; Ct: 0.075 ± 0.003 grams, n: 13) (Figure 4).

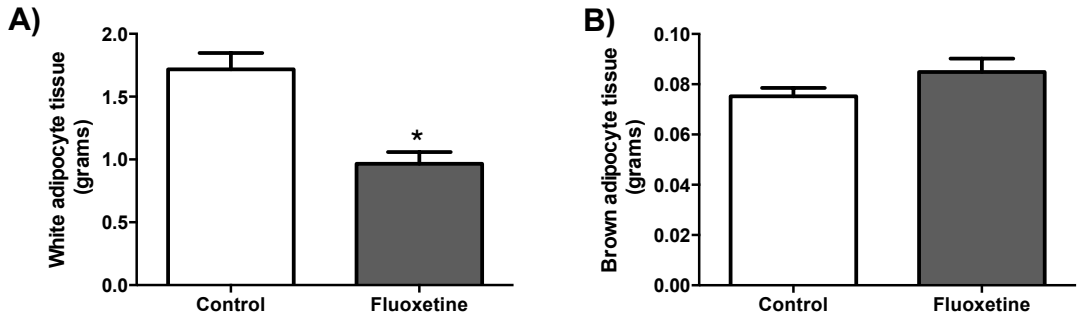

Figure 4 - Effect of neonatal treatment with fluoxetine on White (A) and Brown (B) adipocyte tissue weight (grams) in male rat pups that were treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc) during the suckling period. The retroperitoneal white adipose tissue and brown dorsal tissue weight from the animal on 60 days of life was measured. The adipose tissue weights are presented as the mean ± SEM. (* $p < 0.01$).

3.4 Free Locomotor Activity

Because energy balance is an equation between energy intake and energy expenditure, we evaluated voluntary locomotor activity to verify whether the decrease in body weight and white adipose tissue was because of increased energy expenditure from increased activity. Upon analyzing voluntary locomotor activity for 24 hours, we observed that the Fx group (Fx: 25292 ± 1377 seconds, n: 6) had no significant difference than the C group (C: 27666 ± 1170 seconds, n:6) (Figure 5).

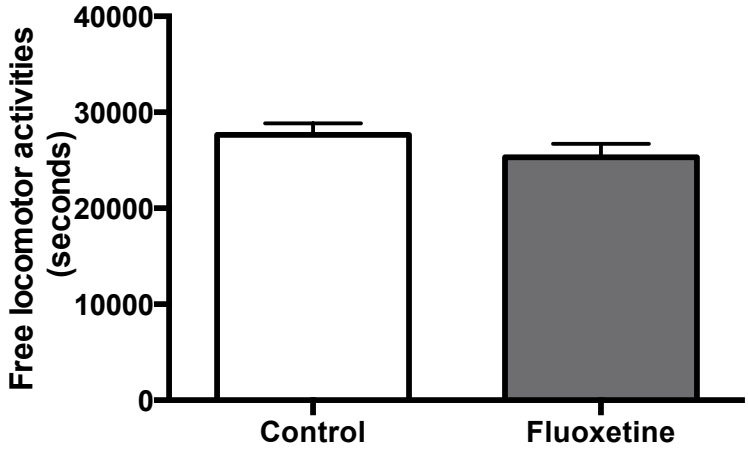

Figure 5 - Effect of neonatal fluoxetine on free locomotor activity (s) in the male pups of rats that were fluoxetine (fluoxetine = 10 mg / kg bw, sc, n = 6) or saline-treated (control = 0.9% NaCl 1 ml/kg, pc, sc, n = 6) during suckling period. The volunteer activities recorded in seconds are presented as the mean ± SEM.

3.5 Temperature variation after thermal stimulation

After evaluating body weight, food intake, adipose tissue and locomotor activity, we were still unable to determine how fluoxetine modulates energy balance. One mechanism that controls energy balance is decreasing metabolic efficiency, in other words, uncoupling substrate oxidation from the production of ATP; this can occur with proton leak that is not coupled to ATP production. In addition, it is well known that cold exposure stimulates lipolysis in BAT; thereby activating UCP1 and increasing heat production (proton transporter). After exposure to -15°C, body temperature was measured using rectal temperature every 30 minutes for a total period of 90 minutes. The Fx group demonstrated a reduced decrease in body temperature (basal temperature= Ct: 33.08 ± 0.44°C, n:5; Fx:32.33 ± 0.65°C, n:6; 30 min after thermal stimulus= Ct: 25.72 ± 0.29°C, n:5; Fx:27.50 ± 0.52°C, n:6; 60 min after thermal stimulus= Ct: 21.66 ± 0.07°C, n:5; Fx:24.58 ± 0.65°C, n:6; 90 min after thermal stimulus= Ct: 18.38 ± 0.17°C, n:5; Fx:23.38 ± 0.12°C, n:6), and the temperature after 90 minutes

demonstrated that the Fx group lost less heat than the Ct group (Fx: 4.56 ± 0.31, n: 5; Ct: 7.42 ± 0.45, n: 6) (Figure 6).

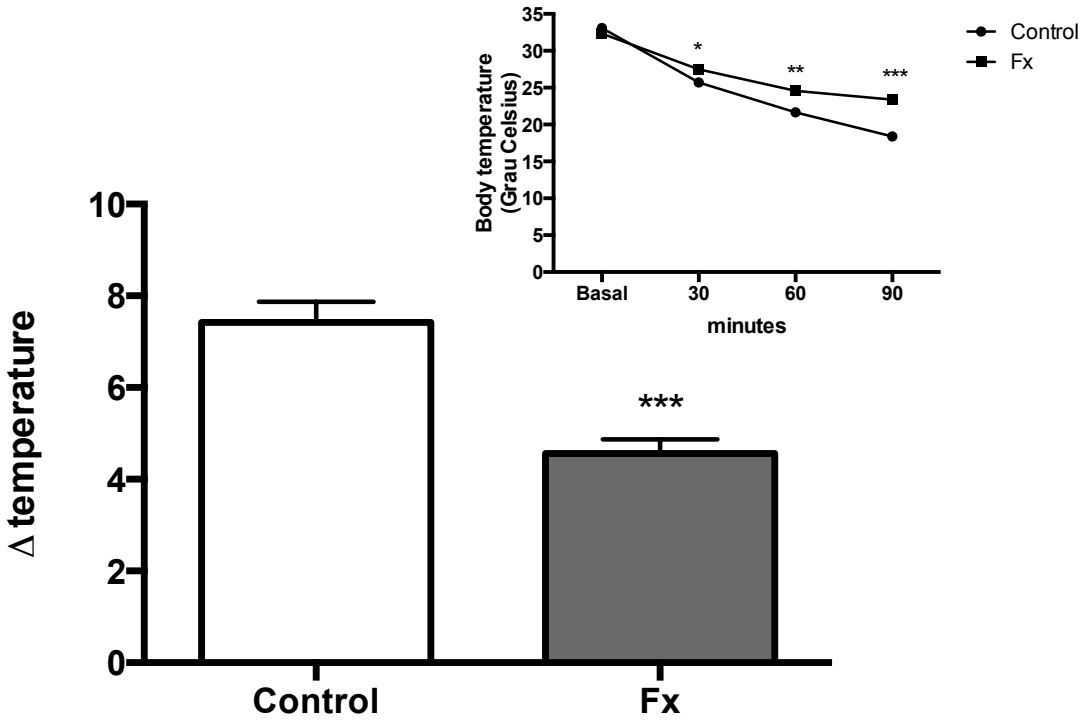

Figure 6 - Effect of neonatal fluoxetine treatment on body temperature in male pups of rats that were treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc, n = 8) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc, n = 8) during suckling period. Body temperature variation was measured progressively every 30 minutes for 90 minutes total. The delta of variation (Δ) in temperature is presented as the mean ± SEM (*p<0.05; **p<0.01;***p < 0.001).

3.6 Mitochondrial oxygen consumption in Brown adipose tissue

Because thermogenesis is mainly a mitochondrial event, mitochondrial function is a good indicator of brown fat activity. To assess the role of BAT mitochondria in the regulation of body weight we examined the mitochondrial oxygen consumption and we observed that mitochondria from Fx group has a higher basal respiration than Ct group (Fx: 56.6 ± 2.13 nmol O_2/min/mg prot, N=11; Ct: 38.0 ± 1.98 nmol O_2/min/mg prot, N=11; p<0.0001); we also observed that after add an uncoupling protonophore (CCCP) the Fx group still showed a higher respiration rate than Ct group (Fx: 71.2 ± 2.27 nmol O_2/min/mg prot, N=11; Ct: 48.0 ± 2.35 nmol O_2/min/mg prot, N=11; p<0.0001) (Figure 7A). These results, together with the temperature led us to the hypothesis that uncoupling proteins could participate in the regulation of the body weight. With this in mind we evaluate the oxygen consumption in BAT

mitochondria in presence and absence of GDP, a potent inhibitor for uncouple protein (UCP 1), and we found a significant decrease of oxygen consumption in Fx group after added GDP (Fx - GDP: 56.6 ± 2.13 nmol O_2/min/mg prot, N=11; Fx +GDP: 46.0 ± 1.7 nmol O_2/min/mg prot, N=6; $p<0.01$) (Figure 7B), adding another piece that suggests an involvement of UCP in Fx-modulating body weight.

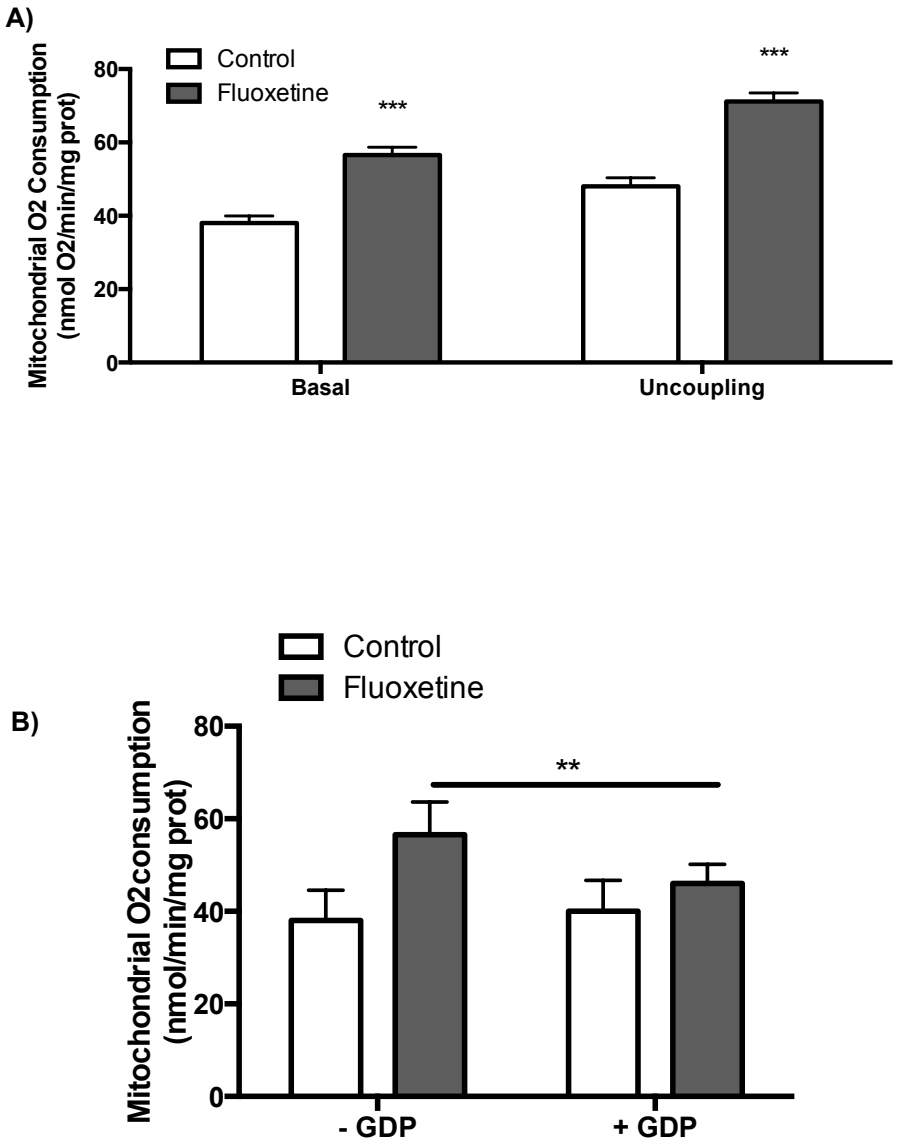

Figure 7 - Effect of neonatal fluoxetine treatment on BAT mitochondrial respiration in male pups of rats that were treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc, n = 11) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc, n = 11) during suckling period. (A) Mitochondrial respiration was measured in State 2 respiration and Maximal respiration capacity (Basal and Uncoupled, respectively); (B) Mitochondrial respiration was measured in absence or presence of GDP during State 2 respiration (n= 6 in each group). The data is presented as the mean ± SEM (**p<0.01;***p < 0.001).

3.7 Mitochondrial ROS production and oxidative stress evaluation

Together, our results suggest the participation of UCP1 in Fx-modulating body weight. We next measure the ROS production from BAT mitochondrial since several studies had shown that mild uncoupling from UCPs could attenuates the production of ROS and oxidative damage [39-41]. As shown in Figure 8, BAT mitochondria from the Fx group produced significantly less ROS than Ct group; consistent with this, we found no difference in oxidative stress evaluated by MDA production and antioxidant enzymes activity in BAT.

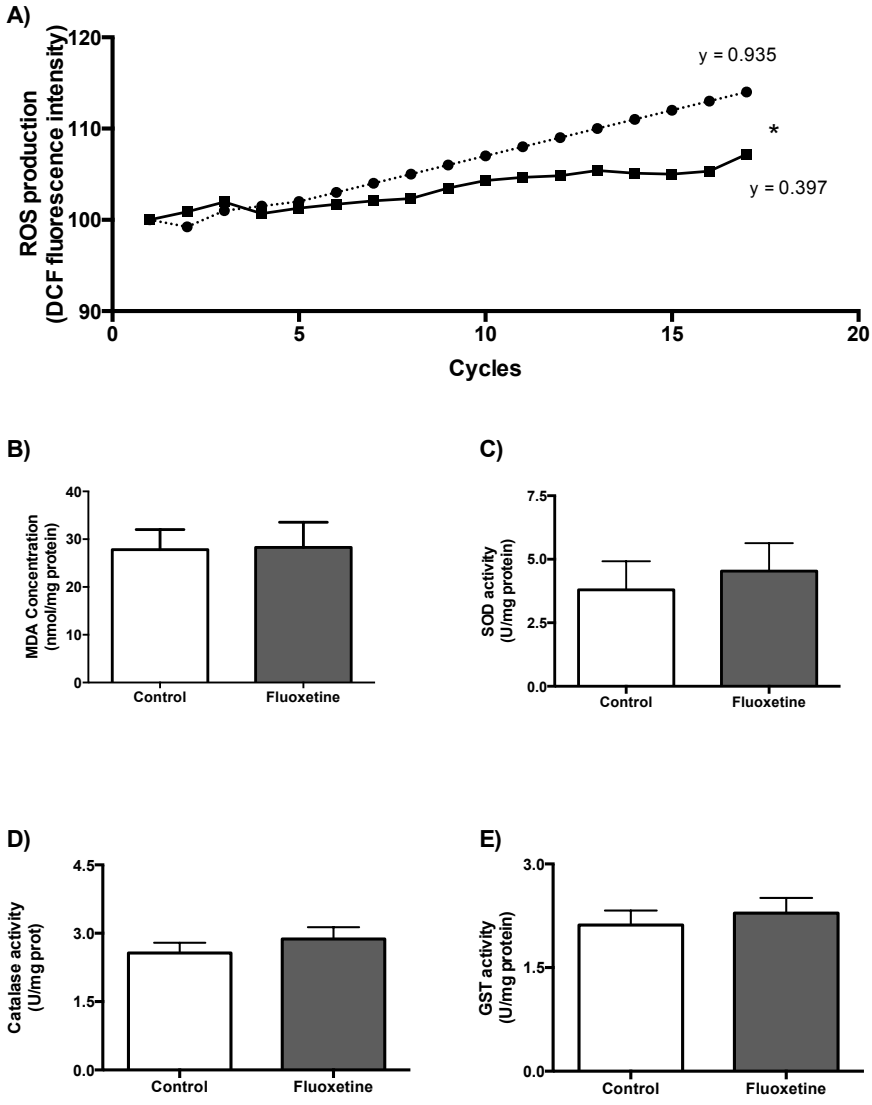

Figure 8 - Effect of neonatal fluoxetine treatment on ROS production and Oxidative stress levels in male pups of rats that were treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc, n = 6) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc, n = 6) during suckling period. (A) Mitochondrial ROS production was performed using DCFDA (1μM); (B) Oxidative stress measured in BAT homogenate. The data is presented as the mean ± SEM (*p<0.05).

3.8 Western blot for UCP 1 expression

To test the direct participation of UCP1 in BAT from the Fx group, we measured the UCP1 protein expression in BAT mitochondria. As shown in Figure 9 neonatal fluoxetine treatment induces an increase in UCP 1 expression (Fx: 2.45 ± 0.07, N=3; Ct 1.98 ± 0.11, N=4; $p<0.05$), suggesting that the decrease observed in body weight may be due to the increase in mitochondrial function especially UCP1 activation. Taking together, our results shown that fluoxetine treatment modulates UCP and improves mitochondrial function.

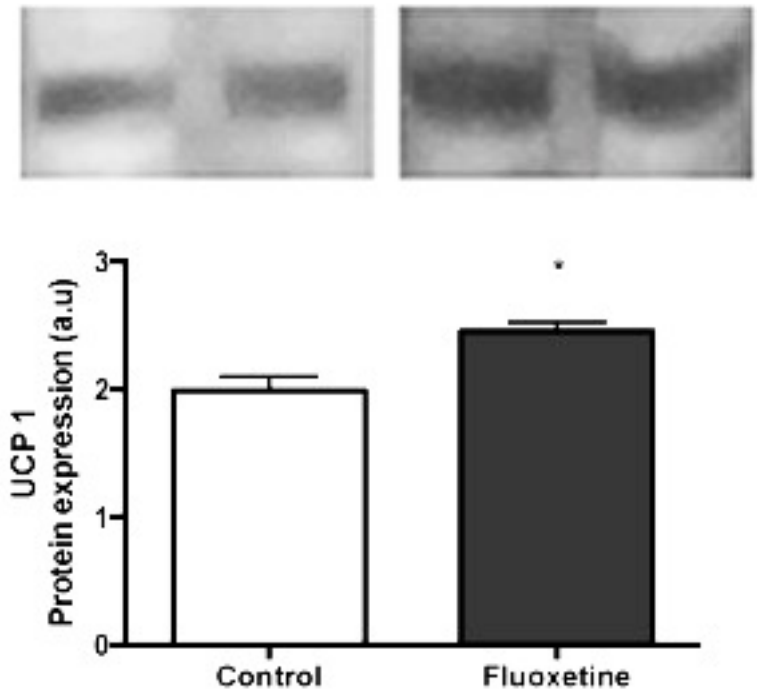

Figure 9 - Effect of neonatal fluoxetine treatment on BAT UCP1 protein expression in male pups of rats that were treated with fluoxetine (fluoxetine = 10 mg / kg bw, sc, n = 3) or saline (control = 0.9% NaCl 1 ml/kg, pc, sc, n = 4) during suckling period. The data is presented as the mean ± SEM (*$p<0.05$).

4. DISCUSSION

In the present study, we demonstrated that chronic treatment (21 consecutive days) with selective serotonin reuptake inhibitors during the period of brain development reduced body weight due to mitochondrial UCP activation, and mitochondrial bioenergetics. In our study, fluoxetine treatment did not induce differences in food consumption compared with controls during the neonatal (7, 14 and 21 days) period or at 40 days for 24 hours of analysis. Previous studies have shown that fluoxetine decreased body weight by anticipate satiety [42, 43], although using our experimental model, we did not observe a difference in food intake.

However, these previous studies were conducted in adult animals using SSRIs such as citalopram, sertraline, fluoxetine or 5-HT or its precursor, the amino acid tryptophan [43, 44]. It is important to highlight that our treatment was conducted during brain development (neonates), and the effects of treatment can be very different between ages and/or developmental stages. The window for nervous system maturation is until the 40th postnatal day, thus these stages are vulnerable to neurobiological changes [45, 46]. It is likely that fluoxetine handling is crucial to the development of specific adaptive behaviors, as our results demonstrate no difference in food intake; drug concentration and treatment duration are variables that affect the response of fluoxetine on food intake.

In agreement with our study, Silva et al. [9] and da Silva et al. [22], using the same experimental model, demonstrates that chronic fluoxetine treatment induced less weight gain than the control during lactation and maintain in adult life. Another study using the antidepressant citalopram (10 mg / kg bw, sc) also demonstrated reduced body weight [47]. The antidepressant fenfluramine (reuptake inhibitor and 5 - HT release stimulator) increased proopiomelanocortin (POMC) expression [48]. The neuropeptide POMC is synthesized in hypothalamic nuclei and emits preganglionic neuron projections in the mediolateral spinal cord; they communicate with skeletal muscle by sympathetic postganglionic fibers [49, 50], which may activate also UCP in skeletal muscle. When this hypothalamic pathway is stimulated, changes in skeletal muscle energy metabolism can occur resulting in increased energy expenditure and decreased body weight [51].

The dissipated energy comprises the basal metabolism, mechanical work (locomotor activity) and adaptive thermogenesis [52]. Thus, high locomotor activity likely increases dissipated energy resulting in body weight loss. Our results showed that fluoxetine treatment has no effect on locomotor activity. However we found alterations in thermal parameters after cold exposure. It is well known that in rodents, cold exposure can sufficiently activate brown adipose tissue (BAT), leading to increased levels of nonshivering thermogenesis via activation of the sympathetic system, and we observe that in the first 30 minutes in cold stimulus the temperature was significant difference between groups. In agreement with our data, studies with rats using another SSRIs, sibutramine, reported increased dissipated energy and reduced body weight [53-57].

Brown adipose tissue (BAT) is the main organ for adaptive thermogenesis in small mammals [58, 59]. Drugs that increase thermogenesis (by activating in BAT) may be possible drug targets for obesity treatment [52, 60]. Activation of β3-adrenergic receptors in brown adipocytes causes lipolysis, increased UCP-1 activity, and thermogenesis [59, 61]. Because

thermogenesis in BAT is a process of metabolic regulation that is controlled by the hypothalamus via descending sympathetic fibers [58], serotonin reuptake inhibitors may act both centrally and peripherally to influence body temperature control [53]. Increasing extracellular 5-HT using reuptake inhibitors may indirectly increase β-adrenergic receptor activation and affect neuronal activity in the melanocortin system [62]. Studies in obese mice treated with leptin demonstrated an increase in hypothalamic and brainstem serotonin, decrease in body weight, and increase in rectal temperature and UCP expression in BAT, suggesting the indirect effect of serotonin in BAT and UCP [63]. Our results related to UCP1 expression and mitochondrial function suggests that neonatal fluoxetine-treatment modulates body weight by increases in UCP1 activation.

This study provides additional information related to the mechanism by which selective serotonin reuptake inhibitors cause weight loss and provides additional insight into the role of serotonin in energy balance regulation. This study could have important clinical implications for understanding new model for obesity treatment because obesity is a leading risk of global death.

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REFERENCES:

[1] K.J. Simansky, Serotonergic control of the organization of feeding and satiety, Behav Brain Res, 73 (1996) 37-42.

[2] L.K. Heisler, E.E. Jobst, G.M. Sutton, L. Zhou, E. Borok, Z. Thornton-Jones, H.Y. Liu, J.M. Zigman, N. Balthasar, T. Kishi, C.E. Lee, C.J. Aschkenasi, C.Y. Zhang, J. Yu, O. Boss, K.G. Mountjoy, P.G. Clifton, B.B. Lowell, J.M. Friedman, T. Horvath, A.A. Butler, J.K. Elmquist, M.A. Cowley, Serotonin reciprocally regulates melanocortin neurons to modulate food intake, Neuron, 51 (2006) 239-249.

[3] M.H. Vickers, P.D. Gluckman, A.H. Coveny, P.L. Hofman, W.S. Cutfield, A. Gertler, B.H. Breier, M. Harris, The effect of neonatal leptin treatment on postnatal weight gain in male rats is dependent on maternal nutritional status during pregnancy, Endocrinology, 149 (2008) 1906-1913.

[4] D.I. Nasyrova, A. Sapronova, A.V. Balbashev, V.S. Kudrin, P.M. Klodt, K.S. Raevskii, M.V. Ugriumov, [Development of central and peripheral serotonin-producing systems in rats in ontogenesis], Zh Evol Biokhim Fiziol, 45 (2009) 68-74.

[5] T.C. Deiro, J. Carvalho, E. Nascimento, J.M. Medeiros, F. Cajuhi, K.N. Ferraz-Pereira, R. Manhaes-de-Castro, Neonatal exposure to citalopram, a serotonin selective reuptake inhibitor, programs a delay in the reflex ontogeny in rats, Arq Neuropsiquiatr, 66 (2008) 736-740.

[6] T.C. Deiro, R. Manhaes-de-Castro, J.E. Cabral-Filho, J.M. Barreto-Medeiros, S.L. Souza, S.M. Marinho, F.M. Castro, A.E. Toscano, R.A. Jesus-Deiro, K.M. Barros, Sertraline delays the somatic growth and reflex ontogeny in neonate rats, Physiol Behav, 87 (2006) 338-344.

[7] J.M. Lauder, H. Krebs, Serotonin as a differentiation signal in early neurogenesis, Dev Neurosci, 1 (1978) 15-30.

[8] C.C. Liao, L.J. Lee, Neonatal fluoxetine exposure affects the action potential properties and dendritic development in cortical subplate neurons of rats, Toxicol Lett, 207 (2011) 314-321.

[9] C.M. Silva, L. Goncalves, R. Manhaes-de-Castro, M.I. Nogueira, Postnatal fluoxetine treatment affects the development of serotonergic neurons in rats, Neurosci Lett, 483 (2010) 179-183.

[10] R. Fleschler, M.F. Peskin, Selective serotonin reuptake inhibitors (SSRIs) in pregnancy: a review, MCN Am J Matern Child Nurs, 33 (2008) 355-361; quiz 362-353.

[11] K. Wynne, S. Stanley, B. McGowan, S. Bloom, Appetite control, J Endocrinol, 184 (2005) 291-318.

[12] B. Zafrir, Brown adipose tissue: research milestones of a potential player in human energy balance and obesity, Horm Metab Res, 45 (2013) 774-785.

[13] S.J. Guyenet, M.W. Schwartz, Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the pathogenesis and treatment of obesity, J Clin Endocrinol Metab, 97 (2012) 745-755.

[14] B.A. De Fanti, D.A. Gavel, J.S. Hamilton, B.A. Horwitz, Extracellular hypothalamic serotonin levels after dorsal raphe nuclei stimulation of lean (Fa/Fa) and obese (fa/fa) Zucker rats, Brain Res, 869 (2000) 6-14.

[15] R. Bross, L.J. Hoffer, Fluoxetine increases resting energy expenditure and basal body temperature in humans, Am J Clin Nutr, 61 (1995) 1020-1025.

[16] I.P. Connoley, Y.L. Liu, I. Frost, I.P. Reckless, D.J. Heal, M.J. Stock, Thermogenic effects of sibutramine and its metabolites, Br J Pharmacol, 126 (1999) 1487-1495.

[17] J.R. Lupien, G.A. Bray, Effect of fenfluramine on GDP-binding to brown adipose tissue mitochondria, Pharmacol Biochem Behav, 23 (1985) 509-511.

[18] K. Arase, D.A. York, N.S. Shargill, G.A. Bray, Interaction of adrenalectomy and fenfluramine treatment on body weight, food intake and brown adipose tissue, Physiol Behav, 45 (1989) 557-564.

[19] S.W. Ma, E. Preston, Disparate effects of fenfluramine on thermogenesis in brown adipose tissue in the rat, Can J Physiol Pharmacol, 70 (1992) 214-218.

[20] N.J. Rothwell, R.A. Le Feuvre, Thermogenesis, brown adipose tissue and dexfenfluramine in animal studies, Int J Obes Relat Metab Disord, 16 Suppl 3 (1992) S67-71.

[21] L. Bellinger, C. Lilley, S.C. Langley-Evans, Prenatal exposure to a maternal low-protein diet programmes a preference for high-fat foods in the young adult rat, Br J Nutr, 92 (2004) 513-520.

[22] A.I. da Silva, L.C.M. Galindo, L. Nascimento, C.M. Freitas, R. Manhaes-de-Castro, C.J. Lagranha, S.L. Souza, Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life, Can J Physiol Pharmacol, (2014).

[23] S. Sanchez, C. Sanchez, S.D. Paredes, J. Cubero, A.B. Rodriguez, C. Barriga, Circadian variations of serotonin in plasma and different brain regions of rats, Mol Cell Biochem, 317 (2008) 105-111.

[24] C. Mendes-da-Silva, S.L. de Souza, J.M. Barreto-Medeiros, S.R. de Freitas-Silva, D.E. Antunes, A.D. Cunha, V.R. Ribas, M.F. de Franca, M.I. Nogueira, R. Manhaes-de-Castro, Neonatal treatment with fluoxetine reduces depressive behavior induced by forced swim in adult rats, Arq Neuropsiquiatr, 60 (2002) 928-931.

[25] K.A. Houpt, T.R. Houpt, Effects of gastric loads and food deprivation on subsequent food intake in suckling rats, J Comp Physiol Psychol, 88 (1975) 764-772.

[26] W.G. Hall, J.S. Rosenblatt, Development of nutritional control of food intake in suckling rat pups, Behav Biol, 24 (1978) 413-427.

[27] B. Grieb, C. von Nicolai, G. Engler, A. Sharott, I. Papageorgiou, W. Hamel, A.K. Engel, C.K. Moll, Decomposition of abnormal free locomotor behavior in a rat model of Parkinson's disease, Front Syst Neurosci, 7 (2013) 95.

[28] S. Li, X. Li, H. Zheng, B. Xie, K.R. Bidasee, G.J. Rozanski, Pro-oxidant effect of transforming growth factor- beta1 mediates contractile dysfunction in rat ventricular myocytes, Cardiovasc Res, 77 (2008) 107-117.

[29] C.J. Lagranha, A. Deschamps, A. Aponte, C. Steenbergen, E. Murphy, Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females, Circ Res, 106 (2010) 1681-1691.

[30] L. Nascimento, C.M. Freitas, R. Silva-Filho, A.C. Leite, A.B. SILVA, A.I. da Silva, D.J. Ferreira, A.A. Pedroza, M.B.S. Maia, M.P. Fernandes, C. Lagranha, The effect of maternal low-protein diet on the heart of adult-offspring: Role of mitochondria and oxidative stress, Applied Physiology, Nutrition, and Metabolism, (2014).

[31] C. Garcia-Ruiz, A. Colell, A. Morales, N. Kaplowitz, J.C. Fernandez-Checa, Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-kappa B: studies with isolated mitochondria and rat hepatocytes, Mol Pharmacol, 48 (1995) 825-834.

[32] H.H. Draper, E.J. Squires, H. Mahmoodi, J. Wu, S. Agarwal, M. Hadley, A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials, Free Radic Biol Med, 15 (1993) 353-363.

[33] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J Biol Chem, 247 (1972) 3170-3175.

[34] H.D. Cardoso, P.P. Passos, C.J. Lagranha, A.C. Ferraz, E.F. Santos Junior, R.S. Oliveira, P.E. Oliveira, C. Santos Rde, D.F. Santana, J.M. Borba, A.P. Rocha-de-Melo, R.C. Guedes, D.M. Navarro, G.K. Santos, R. Borner, C.W. Picanco-Diniz, E.I. Beltrao, J.F. Silva, M.C. Rodrigues, B.L. Andrade da Costa, Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids, Front Hum Neurosci, 6 (2012) 249.

[35] H. Aebi, Catalase in vitro, Methods Enzymol, 105 (1984) 121-126.

[36] W.H. Habig, M.J. Pabst, G. Fleischner, Z. Gatmaitan, I.M. Arias, W.B. Jakoby, The identity of glutathione S-transferase B with ligandin, a major binding protein of liver, Proc Natl Acad Sci U S A, 71 (1974) 3879-3882.

[37] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc Natl Acad Sci U S A, 76 (1979) 4350-4354.

[38] C.J. Lagranha, S.M. Hirabara, R. Curi, T.C. Pithon-Curi, Glutamine supplementation prevents exercise-induced neutrophil apoptosis and reduces p38 MAPK and JNK phosphorylation and p53 and caspase 3 expression, Cell Biochem Funct, 25 (2007) 563-569.

[39] A. Dlaskova, T. Spacek, E. Skobisova, J. Santorova, P. Jezek, Certain aspects of uncoupling due to mitochondrial uncoupling proteins in vitro and in vivo, Biochim Biophys Acta, 1757 (2006) 467-473.

[40] A. Dlaskova, K.J. Clarke, R.K. Porter, The role of UCP 1 in production of reactive oxygen species by mitochondria isolated from brown adipose tissue, Biochim Biophys Acta, 1797 (2010) 1470-1476.

[41] R. Oelkrug, M. Kutschke, C.W. Meyer, G. Heldmaier, M. Jastroch, Uncoupling protein 1 decreases superoxide production in brown adipose tissue mitochondria, J Biol Chem, 285 (2010) 21961-21968.

[42] S.F. Leibowitz, J.T. Alexander, Hypothalamic serotonin in control of eating behavior, meal size, and body weight, Biol Psychiatry, 44 (1998) 851-864.

[43] J. McGuirk, R. Muscat, P. Willner, Effects of chronically administered fluoxetine and fenfluramine on food intake, body weight and the behavioural satiety sequence, Psychopharmacology (Berl), 106 (1992) 401-407.

[44] J.E. Blundell, C.J. Latham, Serotonergic influences on food intake: effect of 5-hydroxytryptophan on parameters of feeding behaviour in deprived and free-feeding rats, Pharmacol Biochem Behav, 11 (1979) 431-437.

[45] S.R. Hansson, E. Mezey, B.J. Hoffman, Serotonin transporter messenger RNA in the developing rat brain: early expression in serotonergic neurons and transient expression in non-serotonergic neurons, Neuroscience, 83 (1998) 1185-1201.

[46] F.C. Zhou, Y. Sari, J.K. Zhang, Expression of serotonin transporter protein in developing rat brain, Brain Res Dev Brain Res, 119 (2000) 33-45.

[47] C.P. Magalhaes, L.O. de Lima, M.C. da Silva, S.M. Marinho, E. do Nascimento, C.M. da Silva, S.L. de Souza, R. Manhaes-de-Castro, [Neonatal treatment effect with selective inhibitor of 5-HT recapture on [corrected] the cranium-encephalic anatomic development], Arq Neuropsiquiatr, 64 (2006) 990-993.

[48] L.K. Heisler, M.A. Cowley, L.H. Tecott, W. Fan, M.J. Low, J.L. Smart, M. Rubinstein, J.B. Tatro, J.N. Marcus, H. Holstege, C.E. Lee, R.D. Cone, J.K. Elmquist, Activation of central melanocortin pathways by fenfluramine, Science, 297 (2002) 609-611.

[49] D.F. Cechetto, C.B. Saper, Neurochemical organization of the hypothalamic projection to the spinal cord in the rat, J Comp Neurol, 272 (1988) 579-604.

[50] C. Broberger, Brain regulation of food intake and appetite: molecules and networks, J Intern Med, 258 (2005) 301-327.

[51] E. Angiolini, A. Fowden, P. Coan, I. Sandovici, P. Smith, W. Dean, G. Burton, B. Tycko, W. Reik, C. Sibley, M. Constancia, Regulation of placental efficiency for nutrient transport by imprinted genes, Placenta, 27 Suppl A (2006) S98-102.

[52] B.M. Spiegelman, J.S. Flier, Obesity and the regulation of energy balance, Cell, 104 (2001) 531-543.

[53] Y.L. Liu, I.P. Connoley, J. Harrison, D.J. Heal, M.J. Stock, Comparison of the thermogenic and hypophagic effects of sibutramine's metabolite 2 and other monoamine reuptake inhibitors, Eur J Pharmacol, 452 (2002) 49-56.

[54] V. Golozoubova, F. Strauss, K. Malmlof, Locomotion is the major determinant of sibutramine-induced increase in energy expenditure, Pharmacol Biochem Behav, 83 (2006) 517-527.

[55] C. Missale, S.R. Nash, S.W. Robinson, M. Jaber, M.G. Caron, Dopamine receptors: from structure to function, Physiol Rev, 78 (1998) 189-225.

[56] S. Izenwasser, D. French, F.I. Carroll, P.M. Kunko, Continuous infusion of selective dopamine uptake inhibitors or cocaine produces time-dependent changes in rat locomotor activity, Behav Brain Res, 99 (1999) 201-208.

[57] H.A. Mitchell, T.H. Ahern, L.C. Liles, M.A. Javors, D. Weinshenker, The effects of norepinephrine transporter inactivation on locomotor activity in mice, Biol Psychiatry, 60 (2006) 1046-1052.

[58] B.B. Lowell, B.M. Spiegelman, Towards a molecular understanding of adaptive thermogenesis, Nature, 404 (2000) 652-660.

[59] A.S. Avram, M.M. Avram, W.D. James, Subcutaneous fat in normal and diseased states: 2. Anatomy and physiology of white and brown adipose tissue, J Am Acad Dermatol, 53 (2005) 671-683.

[60] G.C. Major, E. Doucet, P. Trayhurn, A. Astrup, A. Tremblay, Clinical significance of adaptive thermogenesis, Int J Obes (Lond), 31 (2007) 204-212.

[61] W. Fan, A. Voss-Andreae, W.H. Cao, S.F. Morrison, Regulation of thermogenesis by the central melanocortin system, Peptides, 26 (2005) 1800-1813.

[62] C.S. Myung, B.T. Kim, S.H. Choi, G.Y. Song, S.Y. Lee, J.W. Jahng, Role of neuropeptide Y and proopiomelanocortin in fluoxetine-induced anorexia, Arch Pharm Res, 28 (2005) 716-721.

[63] R.B. Harris, J. Zhou, S.M. Redmann, Jr., G.N. Smagin, S.R. Smith, E. Rodgers, J.J. Zachwieja, A leptin dose-response study in obese (ob/ob) and lean (+/?) mice, Endocrinology, 139 (1998) 8-19.

ANEXOS

Anexo 1 – Comitê de Ética Animal

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Recife, 02 de maio de 2012.

Ofício nº 434/12

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.Raul Manhães de Castro**
Departamento de Nutrição
Universidade Federal de Pernambuco
Processo nº 23076.015276/2012-56

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"INIBIÇÃO DA RECAPTAÇÃO DE SEROTONINA DURANTE O DESENVOLVIMENTO: UM ESTUDO DO BALANÇO ENERGÉTICO E DA FUNÇÃO MITOCONDRIAL"**.

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Departamento de Nutrição-UFPE; Animais: Ratos; Linhagem: Wistar; Sexo: Machos e Fêmeas; número de animais previsto no protocolo:8 ratas lactantes e 64 filhotes; Peso: Ratas 240-260g e filhotes 6-7g; Idade: Ratas adultas e seus filhotes.

Atenciosamente,

Profª. Maria Teresa Jansem
Presidente do CEEA

Anexo 2- Carta Confirmação de Aceite do Artigo – 1

Canadian Journal of Physiology and Pharmacology - Decision on Manuscript ID cjpp-2013-0321.R1

26-Feb-2014

Dear Dr. Lagranha:

It is a pleasure to accept your manuscript entitled "Fluoxetine treatment of rat neonates significantly reduces oxidative stress in the hippocampus and in behavioral indicators of anxiety later in postnatal life" in its current form for publication in the Canadian Journal of Physiology and Pharmacology. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

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Thank you for your fine contribution. On behalf of the Editors of the Canadian Journal of Physiology and Pharmacology, we look forward to your continued contributions to the Journal.

Sincerely,

Drs. Grant Pierce & Donald Smyth
Editors in Chief, Canadian Journal of Physiology and Pharmacology

Anexo 3- Carta de Confirmação de Aceite do Artigo – 2

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Title: Effect of fluoxetine treatment on mitochondrial bioenergetic in central and peripheral rat tissues
Author(s): da Silva, Aline Isabel; Braz, Glauber Ruda; Silva-Filho, Reginaldo; Pedrosa, Anderson; Ferreira, Diorginis; Manhães de Castro, Raul; Lagranha, Claudia

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Title: Neonatal manipulation of the serotonin alters energy balance: Participation of mitochondria and UCP in brown fat tissue
Article Type: Regular Paper
Corresponding Author: Dr. Cláudia J Lagranha
All Authors: Aline Isabel da Silva; Glauber Ruda F Braz; Anderson A Pedroza; Cristiane M Freitas; Raul Manhães de Castro; Cláudia J Lagranha, PhD

Dear Dr. Lagranha,

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