UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

ISOLAMENTO SOCIAL NA PRÉ-PUBERDADE COMO FATOR PROGRAMADOR DO METABOLISMO E INTERAÇÃO COM ALIMENTO CONFORTANTE

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Dedicatória

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SUMÁRIO

APRESENTAÇÃO

Esta tese está organizada em tópicos, a saber: **Introdução**, **Objetivos**, **Capítulos 1 a 4** (referentes aos artigos publicados e ao manuscrito submetido), **Discussão**, **Conclusões**, **Perspectivas**, **Bibliografia e Anexo**.

A **Introdução** apresenta o embasamento teórico que nos levou a formular a proposta de trabalho. Os **Objetivos** – geral e específicos – estão dispostos no corpo da tese e em maiores detalhes inseridos dentro de cada trabalho científico. Os **Capítulos** contêm os artigos publicados e o manuscrito submetido, realizados durante o período do doutorado. Os trabalhos foram desenvolvidos no Laboratório de Neurobiologia do Estresse (Departamento de Bioquímica, ICBS, UFRGS).

O tópico **Discussão** apresenta uma interpretação geral dos resultados obtidos nos diferentes trabalhos. Nas seções **Conclusões** e **Perspectivas** há uma abordagem geral das conclusões da tese e as possibilidades de futuros trabalhos a partir dos resultados obtidos na presente tese.

A **Bibliografia** contém somente as referências dos trabalhos citados nos tópicos **Introdução** e **Discussão**.

No **Anexo** contém tabelas com o resumo dos achados referente ao metabolismo celular neural.

LISTA DE ABREVIATURAS

$\Delta\psi$ = Potencial de Membrana

ACTH = Hormônio adrenocorticotrópico

ADN = Ácido desoxirribonucleico

AGRP= Proteína relacionada ao gene cutia

ARC= Núcleo arqueado do hipotálamo

ATP= Adenosina Trifosfato

CART= Peptídeo relacionado à cocaína e à anfetamina

CAT = Catalase

COX 1= Citocromo oxidase 1

COX 3= Citocromo oxidase 3

CRH= Hormônio liberador de corticotropina

Cu^{2+}= Íon Cúprico

DPN= Dias pós-natal

ERO= Espécies reativas do oxigênio

ERN= Espécies reativas do nitrogênio

$FADH_2$= Flavina Adenina Dinucleotídeo

Fe^{2+}= Íon Ferroso

GCs= Glicocorticóides

GPx = Glutationa peroxidase

GSH= Glutationa

HHA= Hipotálamo-Hipófise -Adrenal

H_2O_2= Peróxido de Hidrogênio

K^+= Íon Potássio

Mn-SOD= Superóxido Dismutase Dependente de Manganês

Na^{2+}= Íon Sódio

NADH= Nicotinamida adenina dinucleotídeo

NADPH = Nicotinamida adenina dinucleotídeo fosfato reduzido

NPY= Neuropeptídeo Y

$O_2^{\bullet-}$= Radical Ânion Superóxido

$^{\bullet}OH$= Radical Hidroxila

PPAR-γ = Proliferação ativada do peroxissomo

RGs= Receptores de glicocorticoides

RMs= Receptores mineralocorticóides

SNC= Sistema Nervoso Central

SOD = Superóxido dismutase

Resumo

Fatores ambientais em períodos precoces do desenvolvimento podem levar a alterações persistentes no sistema nervoso central e no sistema endócrino-metabólico. O período pré-púbere é uma fase crítica do desenvolvimento, quando o encéfalo passa por diversos processos fundamentais, e intervenções ambientais durante essa fase de intensa maturação cerebral podem influenciar a susceptibilidade a doenças ou a resiliência na idade adulta. O estresse por isolamento social destaca-se como um dos mais potentes estressores durante o desenvolvimento e pode levar a alterações a longo-prazo no comportamento social e cognitivo. O estresse também pode prejudicar o metabolismo oxidativo. Outro fator ambiental importante são os alimentos ricos em carboidratos simples, os quais, por um lado, são "alimentos confortantes", ou seja, redutores dos efeitos do estresse, mas também podem desencadear alterações no metabolismo periférico e celular neural. Com base no exposto acima, o objetivo desta tese foi investigar os efeitos do estresse por isolamento social com acesso ou não a um alimento palatável no período pré-púbere (durante o 21-28 dia pós-natal) sobre aspectos metabólicos e neuroquímicos, e investigar memória espacial e possíveis alterações celulares no córtex pré-frontal e no hipocampo de ratos juvenis e adultos. Para alcançar esse objetivo, foram avaliados consumo calórico, parâmetros metabólicos, e o neuropeptideo Y hipotalâmico em ratos jovens e adultos, verificando possíveis mudanças sexo-específicas. Também foram analisados parâmetros do metabolismo energético, do estresse oxidativo, índice de fragmentação do ADN celular e morte celular no hipocampo e no córtex pré-frontal de ratos machos juvenis e adultos. A fim de tentar verificar se as alterações encontradas no metabolismo celular poderiam afetar o comportamento, foram avaliadas a memória espacial e a atividade motora apenas de ratos adultos. Os resultados mostraram que as fêmeas, mesmo antes da puberdade, foram mais propensas a utilizar alimentos confortantes quando expostas ao estresse. Já ratos machos foram mais propensos à programação metabólica a longo-prazo, induzida pela exposição precoce ao alimento palatável, possivelmente relacionada à hipoadiponectinemia. Ambas as estruturas encefálicas estudadas, apresentaram uma programação no metabolismo celular neural a longo-prazo. O isolamento social induziu desequilíbrio entre os sistemas antioxidantes, e induziu uma redução da atividade da enzima Na^+,K^+-ATPase no córtex pré-frontal. A exposição precoce à dieta palatável não foi capaz de prevenir esses efeitos a longo-prazo. No hipocampo de ratos jovens, o isolamento social induziu desequilíbrio oxidativo, aumentou o potencial mitocondrial, o índice de fragmentação ao ADN e a apoptose. Diferentemente dos achados no córtex pré-frontal, o consumo da dieta palatável aumentou atividade de enzimas antioxidantes e preveniu diversos dos efeitos do estresse no hipocampo de ratos jovens. Nos ratos adultos, o isolamento social aplicado no período pré-púbere levou ao desequilíbrio oxidativo a longo-prazo. Houve também, um aumento no número de células necróticas no hipocampo desses animais. A ingestão de alimento palatável durante a exposição ao estresse atenuou alguns efeitos do estresse, incluindo as alterações nas atividades dos complexos respiratórios, no índice de fragmentação do ADN celular e no número de células necróticas. Esses efeitos podem estar relacionados com o aumento no imunoconteúdo de receptores de glicocorticoides no hipocampo, que observamos ter sido induzido pela dieta palatável. No entanto, a essa dieta induziu algumas alterações no metabolismo celular neural, *per se*, aumentando o índice de fragmentação ao ADN celular, o número de células em apoptose inicial e diminuindo o número de células vivas. De modo interessante, não houve diferenças com relação à memória espacial ou à

atividade motora. De uma forma geral, esta tese mostrou que intervenções precoces durante o desenvolvimento do animal podem programar o metabolismo ao longo da vida. Os machos foram mais susceptíveis aos efeitos induzidos pelo consumo precoce de alimento palatável quando avaliados parâmetros do metabolismo a longo-prazo. Com relação aos achados no metabolismo neural, diferenças em resposta à exposição ao estresse e à dieta palatável foram observadas no hipocampo e no córtex pré-frontal. O hipocampo mostrou-se mais susceptível a influências de fatores ambientais precoces. Houve claros efeitos sobre o equilíbrio oxidativo e a função mitocondrial mesmo na idade adulta, muito tempo após as intervenções terem cessado; a dieta palatável foi capaz de prevenir vários dos efeitos do estresse no período pré-púbere.

Abstract

The influence of environmental factors during early periods of development may lead to persistent changes in the central nervous system and in the endocrine-metabolic system. The pre-puberty is a critical stage of development, because the brain is undergoing many fundamental processes and functional organization. Environmental interventions during these periods of intense brain maturation may influence disease susceptibility or resilience in adulthood. Social isolation is one of the most potent stressors during development, and can lead to long-term changes in social behavior, cognitive behavior, and also changes in metabolic parameters. Another important environmental factor that may affect development are foods rich in simple carbohydrates, which are known as comfort foods, since they are able to reduce the effects of stress, however they may as well change peripheral and neural metabolism. Based on the above, the aim of this thesis was to investigate the effects of stress by social isolation in the pre-puberty (during 21-28 postnatal day), with or without the access to a palatable food, on metabolic and neurochemical aspects, and to investigate possible spatial memory and cellular changes in the prefrontal cortex and in the hippocampus of juvenile and adult rats. To achieve this goal, we evaluated caloric intake, metabolic parameters and hypothalamic neuropeptide Y in juvenile and adult rats, verifying possible sex-specific differences. Were also analyzed parameters of energy metabolism, oxidative stress, DNA fragmentation index and cell death in the hippocampus and prefrontal cortex of male juvenile and adult rats. In order to verify whether the possible alterations in cellular metabolism could affect behavior we assessed spatial memory and motor activity. With respect to metabolic parameters, we observed that females, even before puberty, were more likely to use comfort foods when exposed to stress. Male rats were more prone to the long-term metabolic programming induced by early exposure to palatable food, possibly related to hypoadiponectinemia. Both brain structures studied presented long-term alterations in neural cell metabolism. Social isolation induced imbalance between antioxidant systems, and a reduction of Na^+, K^+-ATPase activity in the prefrontal cortex. The palatable diet was not able to prevent these long-term effects. In the hippocampus of juveniles, social isolation induced oxidative imbalance, increased the mitochondrial potential, the DNA fragmentation index and apoptosis. Unlike the findings in the prefrontal cortex, in hippocampus the consumption of palatable diet increased activity of antioxidant enzymes and prevented many of the effects of stress in juvenile rats. In hippocampus of adult rats, social isolation in the prepubertal period led to long-term oxidative imbalance, and increased number of necrotic cells. The intake of palatable food during exposure to stress attenuated some of the effects of stress, including changes in the activities of respiratory complexes, the index of cellular DNA fragmentation and the number of necrotic cells. These effects may be related to the increased immunocontent of glucocorticoid receptors in the hippocampus induced by the palatable diet. However, this diet also induced changes *per se* in neural cell metabolism, including increased DNA fragmentation and apoptosis, and reduced number of live cells. Interestingly, there

were no differences on spatial memory and motor activity. Generally, this thesis has shown that early interventions during the pre-pubertal period can program metabolism throughout life. Male rats were more susceptible to consumption of palatable food when long-term metabolic changes were evaluated. Differences in response to stress exposure and palatable diet were observed in hippocampus and prefrontal cortex. The hippocampus was shown to be more susceptible to influences of early environmental factors. There were clear effects on mitochondrial function and oxidative balance in the adulthood, long after the intervention had ceased; the palatable diet was able to prevent many of the effects of stress in the prepubertal period.

INTRODUÇÃO

Fatores ambientais: Programação encefálica e metabólica

Dados da literatura demonstram que a influência de fatores ambientais em períodos precoces do desenvolvimento pode levar a alterações persistentes no sistema nervoso central (MCQUILLEN e FERRIERO, 2004), e no sistema endócrino-metabólico (SCHIMIDT ET AL., 2009). Existem períodos críticos no desenvolvimento, como por exemplo, o período neonatal, a infância e a adolescência, os quais são fases específicas quando processos dependentes da genética e de fatores ambientais interagem para estabelecer características funcionais (CREWS e HODGE, 2007). Durante esses períodos iniciais da vida do indivíduo, o encéfalo está passando por diversos processos fundamentais como organização funcional das redes neurais, proliferação neural, migração, diferenciação, além de gliogênese e mielinização (RICE e BARONE, 2000). Intervenções ambientais precoces durante essas fases de intensa maturação cerebral podem influenciar a susceptibilidade a doenças ou a resiliência na idade adulta (PAUS ET AL., 2008).

Nesse sentido, estudos têm mostrado que abuso na infância ou negligência promovem alterações a longo-prazo na reatividade ao estresse e no desenvolvimento encefálico (HEIM ET AL., 1997; KAUFMAN ET AL., 2000). Adicionalmente, estudos com ressonância magnética demonstram que a exposição a traumas em uma idade precoce pode levar a diversas alterações neuroestruturais, como a diminuição do hipocampo e do corpo caloso. Além disso, o desempenho e o funcionamento cognitivo também são alterados nessa população, podendo-se correlacionar esses achados com um maior risco em desenvolver transtornos psiquiátricos na idade adulta (BRIETZKE ET AL., 2012).

Outros estudos, usando modelos animais, têm demonstrado que a exposição a estressores nas fases iniciais da vida podem alterar a programação neural (ISGOR ET

AL., 2004; MCEWEN, 1999). Um experimento realizado em ratos machos mostrou que marcadores sinápticos, tais como espinofilina e sinaptofisina, estavam reduzidos no córtex pré-frontal de ratos adultos submetidos ao estresse por isolamento social no final do período pré-púbere (LEUSSIS e ANDERSEN 2008; LEUSSIS ET AL., 2008). Essas alterações persistiram por 25 dias após a retirada do estresse (LEUSSIS ET AL., 2008), sugerindo que as alterações morfológicas induzidas pelo estresse no final da pré-puberdade, observadas no córtex pré-frontal, podem ser mais duradouras do que aquelas observadas na idade adulta (RADLEY ET AL., 2005).

Com relação à influência de fatores ambientais e possíveis alterações persistentes no sistema endócrino-metabólico, é conhecido que a prevalência de sobrepeso na pré-puberdade e puberdade está aumentando, e tem sido sugerido que fatores ambientais, como exposição ao estresse e/ou consumo de alimentos ricos em gordura e carboidratos, estejam fortemente implicados nessa epidemia. Um estudo em ratos mostrou que a exposição a um estressor durante as fases iniciais do desenvolvimento leva a uma alteração na distribuição de gordura, aumentando a razão de gordura visceral/subcutânea quando os animais tornaram-se adultos, levando a um maior risco para o desenvolvimento de doenças metabólicas na idade adulta (SCHMIDT ET AL., 2009). O consumo crônico e precoce de alimentos ricos em gordura também pode modificar as respostas homeostáticas dos circuitos neuroendócrinos e promover alterações na idade adulta, como redução de receptores para leptina no hipotálamo e de receptores para glicocorticoides no hipocampo (BOUKOUVALAS ET AL., 2010).

Assim, o desenvolvimento e a gravidade de diversas condições patológicas na vida adulta dependem não só da vulnerabilidade genética do indivíduo, como também da exposição a fatores ambientais adversos e do período de ocorrência do evento ou fator

ambiental (CHARMANDARI ET AL., 2003), destacando-se principalmente os estágios iniciais do desenvolvimento.

O Período Pré-Púbere: Um Alvo Crítico do Estresse Social

O período pré-púbere em roedores é cronologicamente marcado pelo desmame, por volta do vigésimo primeiro dia de idade pós-natal, e termina em torno do trigésimo dia de idade pós-natal. Durante este período do desenvolvimento, os animais estão em constante mudança, sofrendo transformações comportamentais e neurológicas, dentre elas o aumento da força, da função imunológica e da capacidade cognitiva (DAHL, 2004), além da maturação do comportamento social e cognitivo, do comportamento exploratório e do comportamento de brincadeira (KLEIN ET AL., 2010; SISK e FOSTER, 2004). Assim, a pré- puberdade é uma fase crítica para a maturação cerebral, incluindo os circuitos que controlam a homeostase energética e as respostas ao estresse (MCCORMICK e MATHEWS, 2007).

A maturação e a plasticidade durante o desenvolvimento são altamente variáveis em diferentes regiões cerebrais, incluindo aquelas envolvidas no processamento e na regulação do estresse e da emoção: o córtex pré-frontal, por exemplo, possui uma maturação tardia (GOGTAY ET AL., 2004), e o hipocampo apresenta um aumento na neurogênese e densidade dos espinhos dendríticos antes da puberdade, o que diminui na idade adulta (HE e CREWS, 2007; YILDIRIM ET AL., 2008). Além disso, a composição de proteínas sinalizadoras no hipocampo também é alterada entre o desmame e a idade adulta (WEITZDORFER ET AL., 2008). Muitos sistemas de neurotransmissores, incluindo o sistema dopaminérgico e o glutamatérgico, apresentam uma maturação durante estes estágios iniciais do desenvolvimento (SPEAR ET AL.,

2000). O sistema dopaminérgico mesolímbico não está completamente desenvolvido antes do trigésimo quinto dia de idade pós-natal (SPEAR ET AL. 2000).

Experiências adversas e / ou estressantes durante a pré-puberdade podem ter efeitos marcantes e negativos sobre o comportamento social e sobre a cognição, efeitos esses que podem surgir durante a infância e persistir na idade adulta. Estudos sugerem que estressores no início da vida afetam o desempenho cognitivo de ratos (HODES e SHORS, 2005; TSOORY E RICHTER-LEVIN, 2005). Além disso, o estresse durante os 27-29 dias de idade pós-natal (DPN) também induz alterações de longo prazo na fisiologia e morfologia hipocampal. Cabe ressaltar que o hipocampo é uma estrutura cerebral importante para o aprendizado e a memória, e sua fisiologia e morfologia são sensíveis aos efeitos de estressores agudos e crônicos (HOWLAND e WANG, 2008; TSOORY e RICHTER-LEVIN, 2005).

Assim, tem sido avaliado que os efeitos do estresse parecem ser exacerbados durante a pré-puberdade (CHARIL ET AL., 2010), e é importante considerar que muitas das experiências estressantes durante este estágio da vida parecem envolver o contexto social (MC CORMICK ET AL., 2008). Nesse período, quando as interações sociais são necessárias para o desenvolvimento normal da emoção, perturbações no ambiente social têm efeitos significativos no funcionamento de estruturas cerebrais (EINON e MORGAN, 1977) e no possível desenvolvimento de transtornos psiquiátricos na idade adulta (GUTMAN E NEMEROFF, 2003; LAPIZ ET AL., 2003). Dentre as possíveis perturbações que desencadeiam no ambiente social, os estressores destacam-se por alterarem as interações sociais e o comportamento de brincadeira (KLEIN ET AL., 2010). Além disso, o ambiente social é uma fonte de estresse tanto para os seres humanos quanto para os animais roedores, especialmente durante o período pré-púbere.

No ambiente natural, roedores jovens e mais velhos vivem em grupos e exibem altos níveis de comportamento social (MCCORMICK e MATHEWS, 2007; PANKSEPP e LAHVIS, 2007). Interações sociais são recompensadoras (PANKSEPP ET AL., 2007), enquanto que o isolamento social é aversivo e aumenta a atividade do eixo HHA (DOUGLAS ET AL., 2004; MCCORMICK e MATHEWS, 2007).

O estresse social é um dos mais potentes estressores durante o desenvolvimento (ARAKAWA, 2007) e pode levar a longo-prazo, a anormalidade comportamental, incluindo aumento da agressividade (KOIKE ET AL., 2009; PINNA ET AL., 2003; PINNA ET AL., 2009), comportamentos relacionados à ansiedade (PINNA ET AL., 2006; WEI ET AL., 2007), déficits cognitivos (PIBIRI ET AL., 2008), hipoalgesia e hiperlocomoção (MCEWEN ET AL., 2007). Adicionalmente, este tipo de estressor altera a distribuição de gordura e contribui para o aumento de doenças metabólicas (SCHMIDT ET AL., 2009). Alguns estudos também mostraram que a privação do comportamento de brincadeira durante a adolescência está associada com a falta de regulação do sistema opióide (VAN DEN BERG ET AL., 1999, 2000), prejuízo da memória espacial usando a tarefa do labirinto aquático de Morris (FRISONE ET AL., 2002) e da exploração de novas áreas e objetos (EINON e MORGAN, 1977). Além das alterações comportamentais, o estresse social durante o desenvolvimento também induz alterações morfológicas a longo-prazo (LEUSSIS ET AL., 2008; RADLEY ET AL., 2005). As alterações incluem aspectos estruturais de áreas corticais, com redução da densidade sináptica no córtex infralímbico e no giro denteado (LEUSSIS ET AL., 2008), e pode ser sexo-específico, visto que em ratos machos, mas não em ratas fêmeas, ocorreu uma redução na mielinização no córtex pré-frontal (LEUSSIS e ANDERSEN, 2008). Esses efeitos citados acima, no entanto, são resultado de estresse social de longo-prazo, e os estudos em geral avaliam estresse social aplicado desde o desmame até a

idade adulta. São poucos os estudos de eventos estressores sociais sub-agudos durante a pré-puberdade sobre o comportamento e sobre fatores neuroquímicos no sistema nervoso central.

A Resposta ao Estresse

A transição de neonato para a fase adolescente e para a fase adulta envolve significativas modificações funcionais no eixo HHA. De modo interessante, áreas do cérebro que modulam a resposta do eixo HHA, tais como o hipocampo e o córtex pré-frontal (HERMAN ET AL. 2003), apresentam uma maturação durante o período pré-púbere em roedores. Considerando que muitas estruturas cerebrais participam da resposta do eixo HHA, não é surpreendente que estressores durante este período podem afetar a funcionalidade do eixo HHA e levar a alterações a longo-prazo na reatividade do eixo HHA (FELDON, 2005; MATHEWS, 2002; ROMEO ET AL., 2009). Além disso, hormônios produzidos pelo eixo HHA são importantes mediadores da transição física e fisiológica no desenvolvimento (WADA, 2008).

A exposição ao estresse induz uma variedade de respostas no organismo, incluindo respostas autonômicas, viscerais, imunológicas e neurocomportamentais (ansiedade, depressão e anorexia podem surgir), além da ativação do eixo límbico-hipotálamo-hipófise-adrenal (eixo HHA). A atividade do eixo HHA é regulada pela secreção do hormônio liberador de corticotropina (CRH) pelo hipotálamo, que por sua vez ativa a liberação do hormônio adrenocorticotrópico (ACTH) pela hipófise anterior. O ACTH estimula a secreção de glicocorticóides (GCs; cortisol em humanos e corticosterona em roedores) pelo córtex adrenal. Os glicocorticóides são uma família de hormônios esteróides altamente lipossolúveis que são liberados do córtex adrenal sob o

controle hierárquico do eixo HHA, quando os animais são submetidos a episódios de estresse (SAPOLSKY, 2000). Os glicocorticóides estão envolvidos em vários eventos celulares no SNC, como por exemplo, na plasticidade sináptica, na expressão de receptores de neurotransmissores**,** nas ações de neurotoxinas e no processamento de proteínas (MCEWEN, 2007; MCEWEN e GIANAROS, 2011). Além disso, os glicocorticóides estão envolvidos em transtornos psiquiátricos, tais como depressão e estresse pós-traumático (SAPOLSKY, 2000). As ações desses hormônios são mediadas por ligação aos seus receptores. Classicamente, existem dois tipos de receptores para os glicocorticóides, os quais estão localizados no citosol: os receptores mineralocorticóides (RMs) e os receptores de glicocorticóides (RGs), que podem ser distinguidos pelas diferentes afinidades a seus ligantes. Os RGs estão amplamente expressos no encéfalo, enquanto que os RMs são encontrados primariamente no hipocampo (REUL e DE KLOET, 1985). Esses receptores são ativados na presença dos glicocorticóides e então migram para o núcleo, onde se ligam ao elemento responsivo aos glicocorticóides, presente no ADN, e assim exercem o seu papel transcricional em vários genes.

Um estudo *ex vivo* usando tecido de fígado e de cérebro sugeriu que um pequeno, mas significante, número de RGs pode ser encontrado na mitocôndria, de modo que esta organela pode ser um sítio adicional para as ações diretas dos glicocorticóides (DEMONACOS ET AL., 1996; MOUTSATSOU ET AL., 2001).

Os receptores de glicocorticóides (RGs) estão intimamente envolvidos na retroalimentação negativa dos GCs no eixo HHA após uma situação de estresse **(**DE KLOET ET AL., 2008). Entre as estruturas cerebrais citadas a cima, o hipocampo salienta-se por apresentar um maior número de RGs e por contribuir para a eficiente retroalimentação negativa, diminuindo a resposta do eixo HHA (MCEWEN, 2008; SAPOLSKY, 2003). A hiperatividade deste eixo, indicada por altos níveis de GCs, é

frequentemente observada na depressão **(**FLANDREAU ET AL., 2012; WANG ET AL., 2008**)**. Além disso, um aumento exagerado na expressão ou na função do RG pode estar envolvido no controle do eixo HHA, na resistência ao estresse, na ansiedade e na depressão (ALT ET AL., 2010; DE KLOET ET AL., 2005; RIDDER ET AL., 2005; WANG ET AL., 2012).

Ratos expostos a um estressor agudo durante o período pré-púbere apresentam altos níveis de corticosterona com relação a animais adultos nas mesmas condições (ROMEO ET AL., 2010). Além do mais, animais adultos expostos repetidamente ao mesmo estressor mostram uma habituação na resposta ao estresse com relação aos níveis de ACTH e corticosterona, enquanto que os animais no período pré-púbere exibem uma resposta mais acentuada (DOREMUS- FITZWATER ET AL., 2009; FOLIB ET AL., 2011; ROMEO ET AL., 2006). Alguns estudos têm sugerido uma possível diferença no mecanismo de retroalimentação negativa do eixo HHA ou na expressão dos receptores de glicocorticoides em estruturas cerebrais envolvidas na resposta ao estresse durante o período pré-púbere (GOLDMAN ET AL., 1973, SCHMIDT ET AL., 2007; UYS ET AL., 2006), o que poderia explicar por que ratos, de ambos os sexos, mostram no período pré-púbere, uma resposta mais prolongada na liberação do ACTH e da corticosterona quando submetidos ao estresse com relação aos animais adultos. Ainda, as ratas fêmeas em ambas as idades demonstram um pico maior para resposta a corticosterona, mas retornam aos níveis basais mais rapidamente quando comparadas a ratos machos de ambas as idades (ROMEO ET AL., 2004, a,b).

- Estresse e alimentos palatáveis confortantes

O estímulo crônico do eixo HHA, resultando no excesso de glicocorticóides, parece estar relacionado com ingestão de alimentos altamente palatáveis como forma de

reduzir a resposta do eixo HHA, sendo uma resposta adaptativa ao estresse (PECORARO ET AL., 2004). Contudo, isso mostra que o eixo HHA não só é um condutor das respostas ao estresse como também está intimamente relacionado com a regulação endócrina do apetite (ADAM e EPEL, 2007). Alguns estudos em humanos mostram que indivíduos altamente reativos ao estresse ingerem mais calorias e este comportamento estaria associados com o comportamento compulsivo (EPEL ET AL., 2001; FREEMAN e GIL, 2004). Já estudos em ratos adultos mostram que animais submetidos a um estresse crônico apresentam aumento da ingestão de alimentos palatáveis (ELY ET AL., 1997; PECORARO ET AL., 2004; SILVEIRA ET AL., 2004). A exposição ao estressor é capaz de modificar o comportamento alimentar, que por sua vez, depende da gravidade e da duração do estressor.

A liberação dos glicocorticóides durante situações estressantes pode levar à ação desses hormônios no sistema nervoso central (SNC), possivelmente modulando a ingestão de alimento por meio da ativação do sistema do neuropeptídeo Y (NPY) (DALLMAN ET AL., 1993). A remoção dos glicocorticóides por adrenalectomia suprime o consumo alimentar em 10-20% e diminui o ganho de peso (BHATNAGAR ET AL., 2000), assim como inibe a obesidade induzida pelo NPY (DALLMAN ET AL., 2005). Esses efeitos da adrenalectomia são revertidos pela administração de glicocorticóides (FREEDMAN ET AL., 1985). Como existe uma sobreposição importante em neurônios alvo de glicocorticóides, insulina e leptina, sugere-se que esses hormônios atuem de modo coordenado na regulação do apetite e do gasto energético.

A atividade do eixo HHA, por sua vez, também pode ser influenciada pelo tipo de alimento consumido. Uma dieta hipercalórica, rica em carboidrato e gordura, pode levar a uma redução da resposta do eixo ao estresse (PECORARO ET AL., 2004), sugerindo

um efeito metabólico periférico da dieta sobre o encéfalo (DALLMAN ET AL., 2003). Uma dieta contendo alto teor de gordura, no entanto, realça os níveis de glicocorticóides basais e induzidos por estresse, possivelmente agindo como um fator estressor (KAMARA ET AL., 1998; TANNENBAUM ET AL., 1997).

A ingestão alimentar é regulada por duas vias complementares: (a) homeostática e (b) hedônica. A via homeostática controla o balanço energético por aumentar a motivação para comer quando da redução dos estoques de gordura. A via hedônica, ou regulação baseada na recompensa, pode sobrepor-se à via homeostática durante períodos de relativa abundância de energia, aumentando o desejo de consumir alimentos altamente palatáveis.

O hipotálamo é um importante centro controlador do comportamento alimentar, e também da resposta ao estresse. O núcleo arqueado do hipotálamo (ARC) é uma região posicionada próxima a capilares fenestrados na base do hipotálamo, o que a coloca em contato com importantes hormônios produzidos perifericamente, como leptina (GLAUM ET AL., 1996), insulina (MUROYA ET AL., 1999) e grelina (WANG ET AL., 2002), para os quais possuem receptores (CONE ET AL., 2001). Uma das populações neuronais expressa a proopiomelanocortina (POMC) e o peptídeo relacionado à cocaína e à anfetamina (CART), e quando ativada leva a uma diminuição do apetite e aumento do gasto energético. Em contraste, outra população de células, contendo NPY e Proteína Relacionada ao Gene Cutia (AGRP), leva a uma resposta orexigênica e menor gasto energético (CLARK ET AL., 1984). Tanto os neurônios contendo POMC quanto os neurônios contendo NPY expressam receptores para leptina e grelina (BASKIN ET AL., 1999). A leptina aumenta a atividade dos neurônios POMC

e inibe neurônios NPY (BASKIN ET AL., 1999), enquanto a grelina age fazendo o oposto (TRAEBERT ET AL., 2002).

Outro hormônio importante para o controle alimentar é a adiponectina, a qual é um membro das proteínas secretadas pelo tecido adiposo e que também está envolvida no comportamento alimentar. Sua concentração é mais baixa em homens, indivíduos obesos, diabéticos e com doenças coronarianas (NISHIZAWA ET AL. 2002). A adiponectina age como um agente sensibilizador da insulina, reduzindo a produção de glicose hepática e aumentando a ação da insulina no fígado (GIL-CAMPOS ET AL. 2004), bem como induzindo redução das concentrações de glicose, ácidos graxos livres e triacilglicerois *in vivo* (TOMAS ET AL. 2002). As concentrações de adiponectina correlacionam-se negativamente com insulina de jejum, glicose e triacilglicerois (TOMAS ET AL. 2002).

Os efeitos do estresse sobre o consumo de alimentos confortantes (“comfort foods”) podem ser sexo-específicos (ELY ET AL., 1997; LIANG, 2007). Já é sabido que distúrbios alimentares são mais frequentes em mulheres (PEBLES, 2006). Estudos anteriores do nosso laboratório também mostraram que ratos machos e fêmeas submetidas ao estresse crônico e que recebem chocolate são diferentemente afetados, pois este alimento age como alimento confortante mais evidentemente em fêmeas (FACHIN ET AL., 2008).

- Estresse e mitocôndria

As mitocôndrias são organelas que têm um papel vital na homeostase celular. Apresentam forma e dimensões distintas, as quais dependem do tecido e do estado metabólico. Duas membranas envolvem a mitocôndria: uma externa, lisa que reveste o espaço intermembranas e outra interna, com múltiplas invaginações, denominadas

cristas mitocondriais. Aderidas às cristas encontra-se uma grande quantidade de proteínas componentes da cadeia transportadora de elétrons e a F_OF_1ATPase, responsáveis por realizar a fosforilação oxidativa, além de inúmeras proteínas transportadoras (DAUM, 1985). A maquinaria da fosforilação oxidativa é responsável pela produção aeróbica de ATP. Além dessa importante função, a mitocôndria também participa de vias biossintéticas e de outros processos, tais como o controle intracelular do metabolismo, sinalização do Ca^{+2}, regulação da termogênese, geração de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN), além de servir como um sensor para apoptose.

Essas organelas desempenham papel fundamental na sobrevivência celular, pois são responsáveis pela maior parte da energia gerada e utilizada pelas células eucarióticas (NICHOLLS e FERGUSON, 2002), que provém da cadeia transportadora de elétrons, ou cadeia respiratória, acoplada à fosforilação oxidativa mitocondrial. A cadeia respiratória é responsável pela gradativa transferência de elétrons oriundos do metabolismo intermediário para a redução do oxigênio e síntese de ATP. Durante este processo, os elétrons oriundos das coenzimas nicotinamida adenina dinucleotídeo (NADH) e flavina adenina dinucleotídeo ($FADH_{2)}$, reduzidas durante o ciclo de Krebs ou em outras reações do catabolismo, são transferidos para os complexos I (NADH-ubiquinona oxidorredutase) e II (Succinato-ubiquinona oxidorredutase) e destes para os complexos III (Ubiquinol-Citocromo *c* redutase) e IV (Citocromo *c* oxidase) de uma maneira gradativa, até o aceptor final de elétrons, o oxigênio molecular, com concomitante formação de água. A passagem de elétrons através dos complexos I, III e IV é acompanhada do bombeamento de prótons da matriz mitocondrial para o espaço intermembranas. Este gradiente eletroquímico, responsável pela formação do potencial

de membrana mitocondrial ($\Delta\psi$), dirige o fluxo de prótons de volta à matriz mitocondrial através da F_OF_1ATPase, que utiliza esta energia para a síntese de ATP (HÜTTEMANN ET AL., 2008).

Devido ao papel crucial que a mitocôndria tem na célula, essa organela é uma das primeiras a responder ao estresse. A resposta adaptativa ao estresse envolve alterações importantes nas funções mitocondriais, na capacidade de ajuste da bioenergética, termogênese e respostas oxidativas e/ou apoptóticas **(**MANOLI ET AL., 2007). Dependendo da duração e/ou da intensidade do agente estressor e consequente liberação de glicocorticóides, a resposta mitocondrial pode ser diferente. A resposta mitocondrial envolve, em função das necessidades celulares, sinalização nuclear e mitocondrial destinadas a (1) aumentar o desempenho mitocondrial recrutando um maior número de mitocôndrias (biogênese) ou aumentando o seu volume, (2) melhorar a expressão e a atividade das subunidades da fosforilação oxidativa, (3) possibilitar o desacoplamento da cadeia respiratória, com consequente liberação de energia na forma de calor, (4) facilitar a transdução de sinal para o núcleo e outras organelas intracelulares, (5) gerar ERO para sinalização ou defesa e (6) induzir a cascata de apoptose, dependendo da natureza do estressor (GOLDENTHAL ET AL., 2004).

Estudos mostram que a modulação da atividade mitocondrial pelos glicocorticóides pode ser bifásica. Uma exposição de curto-prazo ao estresse mostra que as concentrações de glicocorticóides são associadas com a indução da biogênese mitocondrial e da atividade enzimática de seletas subunidades dos complexos da cadeia respiratória, enquanto que, se a exposição for mais prolongada, os glicocorticóides podem causar disfunção na cadeia respiratória, aumento na geração de ERO, anormalidades na estrutura mitocondrial, apoptose e morte celular (ALESCI ET AL.,

2006; DUCLOS ET AL., 2004; MANOLI ET AL., 2005; ORZECHOWSKI ET AL., 2002). Um estudo mostrou que o estresse crônico em ratos induz disfunção mitocondrial com alteração na cadeia transportadora de elétrons, diminuição na produção de ATP, apoptose e morte celular (ALESCI ET AL., 2006). Além desses efeitos dos glicocorticóides, foi demonstrado que seus receptores do tipo RG controlam a respiração e a fosforilação oxidativa por meio da regulação transcricional dos genes da mitocôndria (TSIRIYOTIS ET AL., 1997). Alguns estudos mostraram que os genes da citocromo oxidase 1 (COX1) e citocromo oxidase 3 (COX3) podem ser regulados pelos receptores de glicocorticoides presentes nas mitocôndrias. Ambos, COX1 e COX3, são subunidades catalíticas da citocromo c oxidase, a última enzima da cadeia transportadora de elétrons (DEMONACOS ET AL., 1996; LIANG ET AL., 2006). Outro indício da presença dos RGs na mitocôndria foi um estudo que usou várias linhagens celulares e mostrou a capacidade dos glicocorticoides em induzir apoptose por via mitocondrial (SIONOV ET AL., 2006).

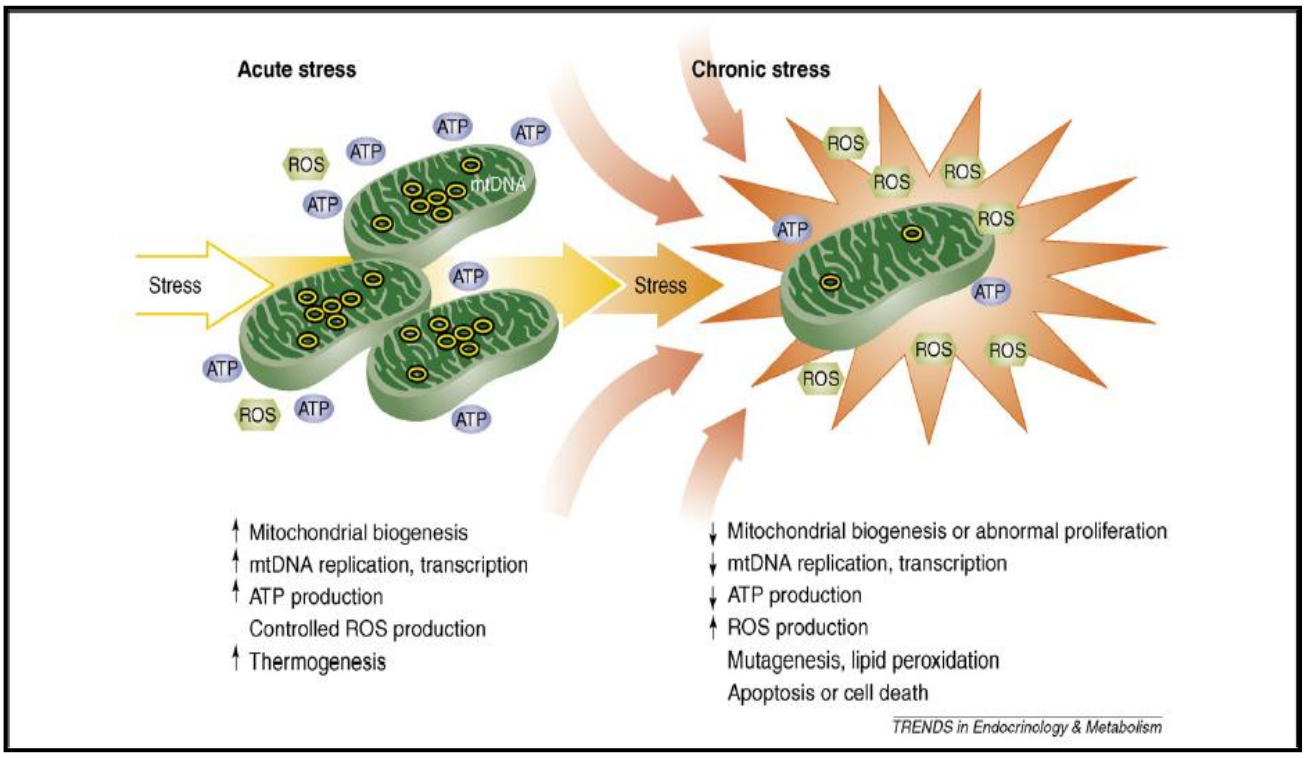

Figura 1: Demonstrativo das alterações mitocondriais em resposta ao estresse (MANOLI ET AL., 2007).

Estresse oxidativo

A cadeia de transporte de elétrons mitocondrial é responsável por gerar espécies reativas do oxigênio e do nitrogênio (DUDKINA ET AL., 2008). Em condições fisiológicas, aproximadamente 1 a 4% dos elétrons é desviado do fluxo pelos complexos da cadeia respiratória, causando a redução incompleta do oxigênio molecular, formando espécies reativas tóxicas como o radical ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\bullet}OH$), o que torna a mitocôndria uma importante fonte de espécies reativas (KOOPMAN ET AL., 2010). A reação do superóxido, $O_2^{\bullet-}$, com o H_2O_2 forma o radical $^{\bullet}OH$, através da reação de Haber-Weiss. A formação de radical hidroxila a partir do peróxido de hidrogênio também pode ser

catalisada pela presença de íons de metais de transição (Fe^{2+} e Cu^{2+}) pela reação de Fenton (GUTTERIDGE e HALLIWELL, 2000; MCCORD, 1987).

No entanto, na tentativa de equilibrar a produção dessas espécies reativas, existem antioxidantes enzimáticos, tais como superóxido dismutase (SOD), glutationa peroxidase (GPx), catalase (CAT) e antioxidantes não-enzimáticos (por exemplo, glutationa [GSH], vitaminas A, C, E, e selênio). O ânion superóxido intramitocondrial, incapaz de se difundir para o citosol, é destoxificado pela enzima antioxidante superóxido dismutase dependente de manganês (Mn-SOD), presente no interior da mitocôndria, gerando peróxido de hidrogênio. Além da Mn-SOD, outros antioxidantes enzimáticos agem na destoxificação do peróxido de hidrogênio (H_2O_2), como a GPx e a CAT. Essas enzimas são responsáveis por degradar o H_2O_2 em água.

A produção dessas espécies reativas pode apresentar um papel benéfico ou prejudicial para célula. Baixas concentrações destas espécies reativas estão envolvidas na regulação de inúmeros processos fisiológicos, tais como a resposta celular a lesões ou infecções, e também na sinalização celular (VALKO ET AL., 2007). No entanto, a superprodução dessas espécies reativas resulta em um dano oxidativo para estruturas celulares, incluindo lipídeos, membranas, proteínas e ADN (VALKO ET AL., 2007). Essa situação é denominada estresse oxidativo, e ocorre quando há um desequilíbrio oxidativo entre os níveis celulares de espécies reativas e de defesas celulares antioxidantes. Esse desequilíbrio pode resultar de um aumento da produção de espécies reativas ou de uma alteração nos mecanismos de defesa, como pela depleção enzimática antioxidante (SOD, GPx e CAT) ou depleção enzimática não-antioxidantes (glutationa, vitaminas e selênio).

Numerosos processos fisiológicos e patológicos, tais como estresse, doenças psiquiátricas, envelhecimento, excesso de ingestão calórica, entre outros, aumentam a concentração dessas substâncias no encéfalo. Cabe ressaltar que o encéfalo é particularmente sensível ao dano oxidativo, pois apresenta modesta capacidade antioxidante e abundante conteúdo lipídico (HALLIWELL, 2006). Além disso, é rico em substratos para oxidação, como, por exemplo, o ferro e o cobre, e requer grandes quantidades de oxigênio para o funcionamento normal, tornando-se um ambiente favorável para o desequilíbrio oxidativo. Tem sido demonstrado que exposição a diferentes tipos de estressores leva a um aumento na produção de ânion superóxido e de peróxido de hidrogênio (WARD e TILL, 1990) e a um desequilíbrio das enzimas antioxidantes, produzindo estresse oxidativo (KOVACHEVA-IVANOVA ET AL., 1994; KROLOW ET AL 2010; MADRIGAL ET AL., 2001; RADAK ET AL., 2001). A exposição a alimentos palatáveis ricos em açúcar e ou gordura pode levar ao estresse oxidativo por um aumento da NADPH-oxidase nos rins e tecido cardíaco e uma redução do sistema antioxidante (LING, 2007). Além disso, dietas ricas em gordura diminuem atividade do receptor gama de proliferação ativada do peroxissomo (PPAR-γ), o qual tem ação anti-inflamatória, e induzem estresse oxidativo. No encéfalo o consumo de alimentos palatáveis pode gerar desequilíbrio oxidativo e dano ao ADN celular em distintas estruturas cerebrais (KROLOW ET AL 2010; OLIVO-MARSTON ET AL., 2008).

Morte celular

As mitocôndrias desempenham um papel central na integração e circulação de sinais iniciadores de morte celular, como o estresse oxidativo e o dano ao ADN celular. Esses sinais, por sua vez, são mediadores-chave nas vias de sinalização que regulam a

apoptose e a necrose celular (ANDREYEV ET AL., 2005; VALKO ET AL., 2006; NIIZUMA ET AL., 2010).

Tem sido sugerido que alterações na fisiologia da mitocôndria e dano celular induzido pela produção de espécies reativas do oxigênio são eventos centrais da apoptose (ANDREYEV ET AL., 2005; VALKO ET AL., 2006; NIIZUMA ET AL., 2010). O processo apoptótico é programado e criticamente controlado por um balanço entre as proteínas pró-apoptóticas e anti-apoptóticas no lado externo da membrana mitocondrial, levando a uma cascata de sinalização que culmina na morte celular (CORY e ADAM 2002; LINDSTEN ET AL 2005). Em resposta ao estímulo de morte, as mitocôndrias são prejudicadas, resultado na liberação de proteínas pró-apoptóticas da mitocôndria para o citoplasma, mas a mitocôndria ainda mantém os níveis de ATP normais, tornando a lesão reversível (Li ET AL., 1997).

A resposta adaptativa ao estresse envolve importantes alterações nas funções mitocondriais permitindo que o organismo ajuste sua bioenergética, termogênese, oxidação e/ ou respostas apoptóticas (MANOLLI ET AL., 2007). Estudos em humanos e também em modelos animais têm proposto que a apoptose é um mecanismo celular que contribui para alterações estruturais observadas em transtornos do humor relacionados ao estresse (MCKERNAN ET AL., 2009). Entretanto, em um dano grave, onde a concentração de ATP encontra-se abaixo dos níveis necessários para o processo de apoptose se instaurar, o dano é irreversível e a morte celular acontece por necrose (LIPTON, 1999). A necrose é um processo patológico e desordenado de morte celular que leva a várias alterações morfológicas, depleção brusca de ATP, perda do controle do balanço iônico, extravasamento do material intracelular devido a um aumento no volume da célula, dano intenso às organelas, ruptura de lisossomos e lise celular (LOCKSHIN e ZAKERI, 2004; YAKOVLEV e FADEN, 2004)

Assim sendo, considerando

- que o período pré-púbere é um período crítico para o estabelecimento de programação metabólica e do funcionamento do sistema nervoso;
- que a ação de fatores ambientais, em especial o estresse e a disponibilidade de dietas altamente palatáveis, que tem aumentado de modo marcante na população nas últimas poucas décadas, poderia afetar de maneira permanente o metabolismo e o comportamento;
- que a função mitocondrial pode ser afetada pela exposição ao estresse e, por sua vez, pode induzir alterações em distintos processos celulares;
- que alimentos confortantes podem reduzir a resposta ao estresse, porém também apresentam efeitos deletérios próprios;
- que é importante considerar diferenças sexo-específicas nas respostas dos organismos ao ambiente, levantamos a hipótese de que a exposição a um estressor durante o período pré-púbere deixaria marcas de longa duração na função encefálica e no comportamento, e considerando ainda que a concomitante disponibilidade de dieta hiperpalatável poderia apresentar interação com os efeitos do estresse, e que tais efeitos poderiam diferir em machos e fêmeas.

OBJETIVOS

OBJETIVO GERAL

O objetivo da presente tese foi investigar os efeitos do estresse por isolamento social e consumo de um alimento palatável no período pré-púbere sobre aspectos metabólicos e neuroquímicos, e investigar memória espacial e possíveis alterações celulares no córtex pré-frontal medial e no hipocampo de ratos jovens e adultos, machos e fêmeas.

OBJETIVOS ESPECÍFICOS

A. Verificar se o estresse por isolamento social durante o período pré-púbere altera o consumo de uma dieta palatável, avaliando o consumo calórico e ganho de peso, e observando se essas condições afetam os parâmetros metabólicos e o NPY hipotalâmico em ratos jovens (28 dias) e adultos (60 dias), observando possíveis diferenças sexo-específico;
B. Investigar os efeitos do estresse por isolamento social e o consumo de dieta palatável no período pré-púbere sobre parâmetros do estresse oxidativo e do metabolismo energético (atividade da Na^+,K^+-ATPase, enzimas da cadeia respiratória, massa e potencial mitocondrial) no córtex pré-frontal medial de ratos machos jovens (28 dias) e adultos (60 dias);
C. Investigar a possibilidade de que o estresse por isolamento social no período pré-púbere leve a alterações celulares (parâmetros do estresse oxidativo, massa e potencial mitocondrial, atividade da cadeia respiratória, apoptose e necrose e índice de quebras ao ADN), no

hipocampo de ratos machos jovens (28 dias) e verificar se o consumo de uma dieta palatável pode reduzir os efeitos do estresse durante o período pré-púbere.

D. Verificar se o estresse por isolamento social no período pré-púbere pode levar a alterações a longo-prazo sobre a memória espacial e a neuroquímica (parâmetros de estresse oxidativo, índice de fragmentação do ADN celular, atividade das enzimas da cadeia respiratória, massa e potencial mitocondrial, apoptose e necrose, BDNF e imunoconteúdo dos receptores de glicocorticoides) hipocampal em ratos machos adultos (60 dias) e verificar se o acesso à dieta palatável durante o mesmo período do desenvolvimento poderia modificar os efeitos do estresse.

MATERIAIS, MÉTODOS

E RESULTADOS

Os materiais e métodos e os resultados desta tese estão apresentados a seguir, da seguinte forma:

- Capítulo 1: Artigo publicado na revista *Metabolism Clinical and Experimental*
- Capítulo 2: Artigo publicado na revista *Neurochemical Research*
- Capítulo 3: Artigo publicado na revista *Neurochemical Research*
- Capítulo 4: Artigo submetido à revista *Pharmacological Biochemistry Behavior*

CAPÍTULO 1

Manuscrito: Sex-specific effects of isolation stress and consumption of Palatable diet during the prepubertal period on metabolic parameters

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Sex-specific effects of isolation stress and consumption of palatable diet during the prepubertal period on metabolic parameters

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ABSTRACT

Objectives. Social isolation during the prepubertal period may have long-term effects on metabolism. The exposure to stressful events is associated with increased palatable food intake, constituting reward-based eating. However, palatable food consumption in early life may lead to metabolic alterations later in life. We investigated whether isolation stress during early life can lead to metabolic alterations in male and female rats with or without exposure to a palatable diet.

Methods. Animals were stressed by isolation during one week after weaning, with or without exposure to a palatable diet.

Results. Stress and palatable diet induced increased caloric consumption. In females, there was a potentiation of consumption in animals exposed to stress and palatable diet, reflected by increased weight gain and triacylglycerol levels in juveniles, as well as increased adiponectin levels. Most of the effects had disappeared in the adults. Different effects were observed in males: in juveniles, stress increased unacylated ghrelin levels, and hypothalamic neuropeptide Y (NPY). Subsequently, adult males that were exposed to a palatable diet during prepuberty showed increased body weight and retroperitoneal fat deposition, increased glycemia, and decreased plasma adiponectin and hypothalamic NPY. Exposure to stress during prepuberty led to increased adrenals during adulthood, decreased LDL-cholesterol and increased triacylglycerol levels.

Conclusion. Isolation stress and consumption of palatable diet changes metabolism in a sex-specific manner. Prepuberty female rats were more prone to stress effects on food consumption, while males showed more long-lasting effects, being more susceptible to a metabolic programming after the consumption of a palatable diet.

Abbreviations: NPY, hypothalamic neuropeptide Y; GCs, glucocorticoids; HPA, hypothalamo-pituitary adrenal axis; PND, postnatal day; HDL, high-density lipoprotein; LDL, low-density lipoprotein; EDTA, Ethylenediamine tetraacetic acid; VLDL, very low density lipoprotein.

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1. Introduction

The prevalence of obesity during prepuberty and puberty is increasing, and environmental factors, such as exposure to stress, are thought to be strongly implicated in this epidemic [1,2]. The prepubertal and pubertal periods are marked by continuous emotional development and great brain plasticity [3]. Interventions during this critical period may have long-term effects on emotion, behavior and metabolism [4,5]. One of the most potent stressors during the peri-pubertal period is social isolation [6], which can lead to behavioral, morphological and neurochemical changes, which may also be present in adulthood [7,8].

Exposure to stressful events leads to an increased release of glucocorticoids (GCs) by activation of the hypothalamo-pituitary adrenal (HPA) axis. Under normal conditions, activation of the stress response, caused by everyday stressors, results in adaptive endocrine, metabolic, and cardiovascular changes that help to maintain homeostasis [4,5]. However, prolonged elevations in glucocorticoid and catecholamine concentrations in the circulation result in changes that may be maladaptive [9,10], affecting the timing of puberty, final stature, and body composition, as well as causing early-onset obesity, metabolic syndrome, and type 2 diabetes mellitus [3]. In addition, one of the consequences of stress exposure is altered food intake [11,12], causing either increased or decreased food intake, depending upon the nature of the stress [13]. Animals that are repeatedly stressed by restraint show increased ingestion of sweet food [11], while models of chronic variate stress induce a decreased appetite for sweet food or palatable solutions [12,14]. Furthermore, there is evidence that glucocorticoids stimulate appetite [15], and increase body weight through the orexigenic effect of the hypothalamic feeding signal, neuropeptide Y (NPY) [16], an effect that is inhibited by leptin and insulin [17]. In addition, excess glucocorticoids increase glucose and insulin and decrease adiponectin levels [18].

Human studies also show greater food consumption, mainly palatable food, during periods of psychological stress [19]. The increased palatable food intake induced by glucocorticoids [20] has been associated with reward-based eating, which has been suggested as a means to reduce the stress response [21], and palatable food, rich in carbohydrates ("comfort food"), has been shown to decrease the stress response in stressed rats [22]. These observations suggest the possibility of an interaction between the effects of stress and consumption of easily available palatable food. Additionally, stress effects on "comfort food" consumption may be sex-specific [10,23]. Moreover, it is known that eating disorders are more frequent in women [24]. It is noteworthy that male and female rats subjected to stress and receiving chocolate as a "comfort food" are differentially affected [25,26], which emphasizes the importance of considering sex-specific differences in studies concerning stress and food consumption.

Although several studies have shown the effects of stress on food consumption, the effects of stress when applied during the prepubertal period and its possible interaction with the consumption of a palatable diet have not been studied, especially when considering sex-specific differences. Therefore, the aim of this study is to verify whether isolation stress during the prepubertal period alters the consumption of a highly palatable diet, evaluating caloric consumption and weight gain, and to observe whether these conditions affect metabolic parameters later on in life, during adulthood. Our hypothesis is that consumption of a palatable diet during the stress period will increase and that access to this type of diet may counterbalance long-term adverse stress effects. Additionally, we also hypothesized that males and females will present different outcomes later in life for these events occurring in the prepubertal period. Body weight and adrenal weight, abdominal fat deposition, the concentration of fuels (glycemia, plasma lipids), and hormones related to fuel homeostasis (insulin, leptin, ghrelin, adiponectin), as well as hypothalamic NPY were measured, using both male and female rats.

2. Methods

2.1. *Subjects*

All animal procedures were approved by the Institutional Ethical Committee (CEUA-UFRGS 20040), and followed the recommendations of the Federation of Brazilian Societies for Experimental Biology. All efforts were made to minimize animal suffering, as well as to reduce the number of animals. Wistar rats were weaned on postnatal day 21. Males and females were separated according to sex and weighed. Half of the animals were housed in groups of 4–5; the other half were stressed by isolation (one animal in a smaller home cage, 27 × 17 × 12 cm) [27]. Each group (control or stressed) was subdivided according to the diet they received: Receiving a high palatable diet or standard lab chow. Animals were divided in such a way that only one animal per litter was used per group. These procedures resulted in four experimental groups: (1) Controls receiving standard lab chow (15 males and 16 females), (2) controls receiving palatable diet (20 males and 16 females), (3) isolated animals receiving lab chow (20 males and 16 females), and (4) isolated animals receiving palatable diet (20 males and 16females). These interventions occurred between postnatal days 21–28 and daily food consumption was measured. At postnatal day 28 (PND 28), half of the animals in each group were killed by decapitation and biochemical evaluations were performed in the plasma and the hypothalamus. The other half of the animals received standard chow and were reared in groups of 5 per cage until adulthood, when they were killed by decapitation and the same biochemical parameters were assessed.

2.2. *Palatable diet*

The palatable diet used on this study was enriched in simple carbohydrates. The diet was composed of condensed milk, sucrose, a vitamin and salt mix, powdered standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet was similar to that of a standard lab chow (including 22–24% protein and 4–6% fat), however 41.4 g% of carbohydrates in the palatable diet were

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represented by 26.7 g% of sucrose and lactose and 14.7 g% of starch; in contrast, the standard lab chow had 49 g% carbohydrates, mainly from starch.

2.3. Food consumption, body weight and weight gain

Previously weighed amounts of standard lab chow and palatable diet were offered and the remaining amounts of pellets were measured each day to evaluate consumption. The food consumption was measured per cage and, in the control cages, the amount of food consumed was divided by the number of animals per cage to determine mean consumption per animal. To verify the amount of kilocalories consumed per animal, the amount of food ingested was multiplied by the caloric content per gram of chow or diet. The standard lab chow has a caloric content of 3.24 kcal/g, whereas the palatable diet has a caloric content of 4.5 kcal/g (being 38% more caloric than the standard chow).

2.4. Abdominal fat and adrenal gland dissection

At PND 60, animals were killed by decapitation after 6h of fasting (7:00–13:00 h). The two major portions of abdominal fat (gonadal and retroperitoneal adipose tissue depots) and adrenal glands were carefully dissected and weighed. Abdominal fat is shown in grams. Adrenal weight is expressed in relation to the body weight.

2.5. Biochemical analysis

2.5.1. Preparation of the samples for biochemical measurements

Trunk blood was collected into tubes with ethylenediamine tetraacetic acid (EDTA) for glucose, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol; leptin, insulin, adiponectin, and unacylated ghrelin determination. Plasma was separated and frozen until the day of analysis.

2.5.2. Plasma glucose and lipid levels

Glucose, triglycerides, and total cholesterol were measured using commercial kits from Wiener Laboratorios (Rosario, Argentina). HDL-cholesterol was measured using a kit from Labtest Diagnóstica S.A. (Minas Gerais, Brazil). LDL-cholesterol was evaluated through the Friedewald formula [28].

2.5.3. Plasma levels of insulin, leptin, adiponectin, and unacylated ghrelin

Plasma insulin (Rat/Mouse insulin ELISA kit Millipore, n° cat ≠ EZRMi-13K), leptin (Leptin clear Microtiter plate Enzo life sciences, n° cat ≠ 80–1793), adiponectin (Rat adiponectin Elisa Assay kit Biovision, n° cat ≠ k4903-100) and desacetylated ghrelin (Rat unacylated Ghrelin Enzyme Imunoassay kit SPI bio, n° cat ≠ A05118) were measured using commercial ELISA kits.

2.5.4. Determination of hypothalamic NPY

The whole hypothalamus was dissected from the brain and the samples were homogenized in Extraction Buffer (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, pH 7.9) with a protease inhibitor (100:1). A detergent (NP40 1%) was then added and the homogenate was centrifuged at 3000×*g* for 10 minutes at 4 °C. The supernatant (cytosolic fraction) was used for NPY quantification later using a commercially available peptide enzyme immunoassay kit (Peninsula Laboratories LCC, CA).

2.5.5. Protein assay

The total protein concentrations were determined by the Lowry method with bovine serum albumin as the standard [29].

2.6. Statistical analysis

Data were expressed as mean ± SE of the mean, and were analyzed using repeated Measures ANOVA (body weight and food consumption), or ANOVA with isolation stress, palatable diet and sex as independent factors. A Duncan post hoc test was performed, if necessary. All analyses were performed with SPSS software and a $P < 0.05$ was considered significant. Regarding Repeated Measures ANOVA, Greenhouse-Greisser correction was applied considering violation of the sphericity assumption as shown by the Mauchly test.

3. Results

3.1. Food consumption, body weight and weight gain

Caloric consumption, body weight and body weight gain were analyzed during the period of stress and up to 60 days of life. The mean consumption for each week was analyzed using repeated measures ANOVA. As displayed in Fig. 1, during the first week (PND 21–28; period of isolation stress), both male and female rats had increased caloric consumption over time [$F(4.24, 331.20) = 25.68$, $P < 0.001$, correction for Greenhouse-Geisser]. Access to a palatable diet increased caloric consumption [$F(1,78) = 126.45$; $P < 0.001$], and the same effect was observed for exposure to isolation stress [$F(1,78) = 21.78$; $P < 0.001$]. An interaction between exposure to stress and access to a palatable diet was observed [$F(1, 78) = 10.35$, $P < 0.002$], since the animals from the group submitted to isolation and receiving a palatable diet showed a potentiation of the effect of both factors on consumption, with a higher increase in the caloric consumption during this period. An interaction between exposure to stress and sex was also observed, since the stress-induced increase in caloric consumption was more marked in females. A Duncan post hoc test was performed to indicate individual differences between the groups, as shown in Fig. 1.

In relation to weight gain during PND 21–28 (Fig. 2), a three-way ANOVA showed that rats that received a palatable diet had a higher weight gain [$F(1,72) = 6.80$; $P < 0.02$]; there was an effect of sex [$F(1,72) = 13.40$; $P < 0.001$], since males gained more weight; in addition, there was an interaction between stress, palatable diet and sex [$F(1,72) = 6.65$; $P < 0.02$], since the group submitted to both stress and palatable diet showed a much higher weight gain, but only in females.

After PND 28, isolated animals were returned to groups of 4–5 per cage and all animals received standard lab chow. Caloric consumption was analyzed (Fig. 3) and the results demonstrate that rats had increased caloric consumption over time [repeated measures ANOVA, $F(1.78, 21.40) = 12.74$,

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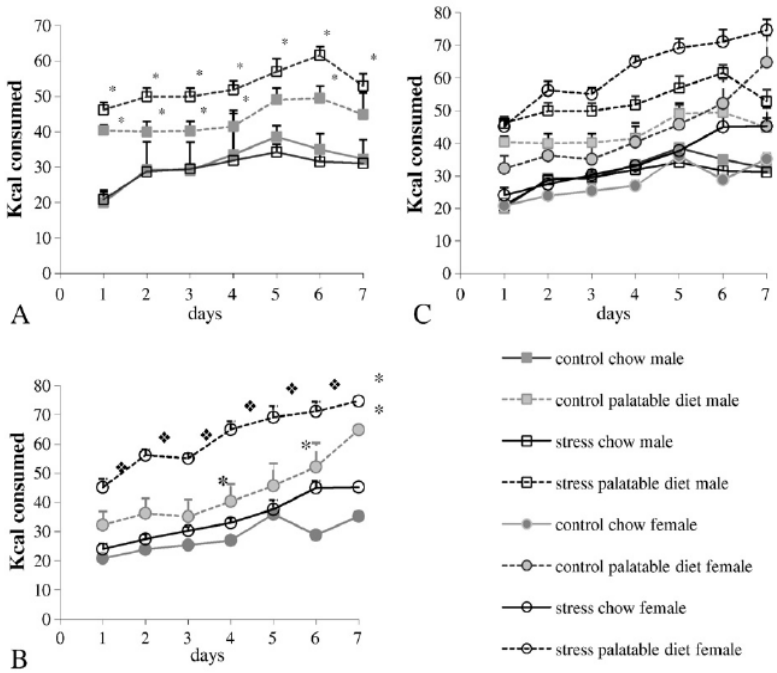

Fig. 1 – Effect of isolation stress during the prepubertal period, with or without access to a palatable diet, on caloric consumption during PND 21–28 in male (A) and female (B) rats; Panel (C) shows consumption from all groups for comparison. Data are expressed as mean ± SEM, N = 4–5 (for cages of animals, when in groups, with 4–5 animals/cage); N = 20 (for isolated animals). The mean consumption was analyzed using repeated measures ANOVA. Both male and female rats increased caloric consumption over time ($P < 0.001$). Access to a palatable diet increased caloric consumption both in male ($P < 0.01$) and female ($P < 0.001$) rats, and the same effect was observed for exposure to isolation stress for male ($P < 0.05$) and female ($P < 0.001$) rats. In females, there was also an interaction between exposure to stress and access to a palatable diet ($P < 0.05$). post hoc results are shown in panels (A) and (B). *Different from respective group receiving standard chow (post hoc Duncan test; n = 5cages with 4 animals/cage for control palatable diet group, n = 20 for stress palatable diet group, for control standard chow group, n = 5 cages with 5 animals/cage). ❖ Different from all other groups (post hoc Duncan test; n = 4 cages with 5 animals/cage for control palatable diet group, n = 16 for stress palatable diet group, for control standard chow group, n = 4 cages with 5 animals/cage, and n = 16 for stress standard chow group).

$P < 0.001$, correction for Greenhouse-Geisser], with an interaction between time and sex, since males showed a greater increase in consumption over time [$F(1.78, 21.40) = 6.00$, $P < 0.02$, correction for Greenhouse-Geisser]. Previous access to a palatable diet increased caloric consumption during this period [$F(1,12) = 5.90$; $P < 0.05$]. When analyzing body weight until adulthood, we observed that there was an increased body weight over time [$F(2.44, 119.74) = 1140.96$, $P < 0.01$, correction for Greenhouse-Geisser]. There was also an interaction between time and sex [males showed a greater increase in weight over time; $F(2.44, 119.74) = 40.93$, $P < 0.001$, correction for Greenhouse-Geisser]. Effects of previous exposure to a palatable diet during the prepubertal period [$F(1,49) = 8.36$; $P < 0.01$], and of sex [$F(1,49) = 37.09$; $P < 0.001$] were also observed (Fig. 4).

3.2. *Abdominal fat and adrenal weight*

With regard to abdominal fat, there were effects of sex both on retroperitoneal fat [$F(1,65) = 23.79$, $P < 0.001$] and on gonadal fat [$F(1,63) = 5.53$, $P < 0.05$], since males had a higher fat deposition. Adult male animals that received the palatable diet during the prepubertal period had an increased deposition of retroperitoneal fat, as shown by an interaction between diet and sex [$F(1,65) = 7.33$, $P < 0.001$]. In addition, the adrenal-gland weight showed an effect of sex [$F(1,66) = 23.87$; $P < 0.001$], since female adrenals had a higher weight. When analyzing both sexes separately, isolation stress in the prepubertal period increased the adrenal-gland weight in adult male rats [$F(1,66) = 13.83$; $P = 0.05$] (Table 1).

3.3. *Plasma lipid levels*

Results from plasma lipid measurements were analyzed using a three-way ANOVA, and are shown in Table 2. In juveniles, exposure to isolation stress in the prepubertal period was able to increase plasma triacylglycerol levels [$F(1,27) = 7.51$, $P < 0.02$]. Stress also increased total cholesterol, but only in animals that received the palatable diet, as shown by an interaction between stress and diet [$F(1,25) = 5.22$, $P < 0.05$]. In

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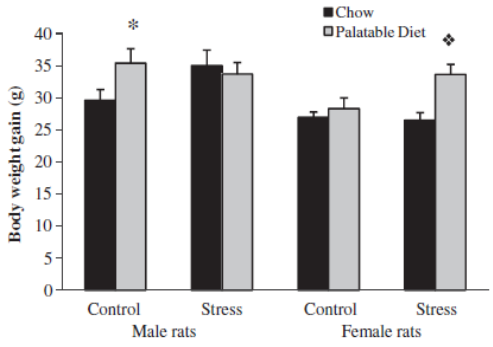

Fig. 2 – Effect of isolation stress during the prepubertal period, with or without access to a palatable diet during this period, on weight gain during PND 21–28, in male (A) and female (B) rats. Data are expressed as mean ± SEM, N = 10/group. Three-way ANOVA showed that female rats that received a palatable diet displayed increased weight gain ($P < 0.01$) and there was an interaction between stress and palatable diet in female ($P < 0.05$) rats. No difference in weight gain during this period was observed in male rats (three-way ANOVA, $P > 0.05$ in every case). *Different from respective group receiving standard chow (post hoc Duncan test). ❖ Different from all other groups (Duncan post hoc test.)

adult animals, stress during the prepubertal period increased plasma levels of triacylglycerols [$F(1,27) = 4.45$, $P < 0.05$]. Isolation stress during the prepubertal period had opposite effects in males and females on total cholesterol and on LDL-cholesterol [interaction stress and sex, $F(1,30) = 14.20$, $P < 0.001$ for total cholesterol, and $F(1,22) = 6.77$, $P < 0.02$ for LDL-cholesterol], since stress increased these parameters in females and decreased them in males, as may be observed in Table 2.

3.4. *Plasma levels of glucose, insulin, leptin, adiponectin, and ghrelin*

Levels of glucose and of hormones related to feeding behavior (insulin, ghrelin, leptin and adiponectin) in juvenile rats are displayed in Table 3. Table 4 presents the levels of these same parameters in adult rats.

In juvenile male rats, a palatable diet decreased plasma levels of insulin, as shown by an interaction between diet and sex [$F(1,29) = 6.08$, $P < 0.02$]; isolation stress showed a marginally significant effect on unacylated ghrelin [$F(1,29) = 3.42$, $P = 0.07$]. There was also an effect of the interaction of isolation, palatable diet and sex on plasma glucose levels in juvenile rats [$F(1,27) = 4.12$, $P = 0.05$], since both factors (stress and diet) induced slight reductions in this parameter, mainly in males, which were not added in the isolated group receiving the palatable diet. In adult rats, a three-way ANOVA showed interactions between stress and palatable diet [$F(1,31) = 5.52$, $P < 0.001$], and between palatable diet and sex [$F(1,31) = 4.45$, $P < 0.05$] on glycemia, since exposure to this diet in the prepubertal period increased glucose levels in adulthood in male animals that had been isolated.

Plasma levels of adiponectin were increased in isolated female juvenile rats, and were decreased in isolated male rats, as shown by an interaction between stress and sex [$F(1,43) = 4.09$, $P < 0.05$]. This stress effect does not appear in adults; however, an effect of sex on adiponectin was observed in adults [$F(1,32) = 4.44$, $P < 0.05$], as well as an effect of exposure to palatable diet during the prepubertal period [$F(1,32) = 5.99$, $P < 0.02$]. Interestingly, when analyzing both sexes separately, decreased plasma levels of adiponectin were observed in adult male rats [$F(1,16) = 6.46$, $P < 0.05$] that had been exposed to the palatable diet during the prepubertal period; this effect was not seen in females. There were no changes in leptin levels ($P > 0.05$).

3.5. *Hypothalamic NPY*

Hypothalamic neuropeptide Y (NPY) levels in male and female rats of different ages were also evaluated (Table 5). Isolation stress in the prepubertal period induced an increase in the hypothalamic NPY only in juvenile male rats, as shown by an interaction between stress and sex [$F(1,37) = 4.31$, $P < 0.05$]. In adult male rats, the palatable diet decreased hypothalamic NPY, as shown by an interaction between exposure to this diet and sex [$F(1,45) = 5.32$, $P < 0.05$].

4. Discussion

This study provides new data regarding differences between the sexes for prepubertal stress effects. Interestingly, although these animals had not yet entered puberty, both sexes reacted differently to stress and to the access to the palatable diet. During PND 21–28 (period of isolation stress), the access to a palatable diet increased caloric consumption, probably as a result of the palatability of this diet. However, stressed female rats with access to the palatable diet showed a potentiation of this effect, with a higher increase in the caloric consumption and in weight gain during this period. Reward-based eating has been proposed as a means to reduce the stress response [20], and our results suggest that female rats in the prepubertal period are more susceptible to the use of a palatable diet as comfort food during periods of stress.

Behavioral and physiological responses to stress are known to be sexually dimorphic. It has been suggested that the female response to stress involves a different pattern of hormones (including oxytocin), and this response was called by Taylor et al. [30] the "tend-and-befriend" response, instead of the "fight-or-flight." This peculiar response would increase the survival of females and their offspring. Concerning feeding behavior, a more marked effect of stress on food consumption in females than in males has been shown in adults [31]. In addition, Fachin et al. [25] showed that chocolate consumption may prevent some effects of chronic stress in females (and not in males). Our results suggest that these sex-specific differences may also be present early on life. Additionally, since animals had not yet reached puberty, this effect does not depend on the circulating levels of gonadal hormones.

Interventions during the prepubertal period may have long-term and, frequently, irreversible effects on behavior and metabolism [4,5]. Therefore, we evaluated caloric consumption after

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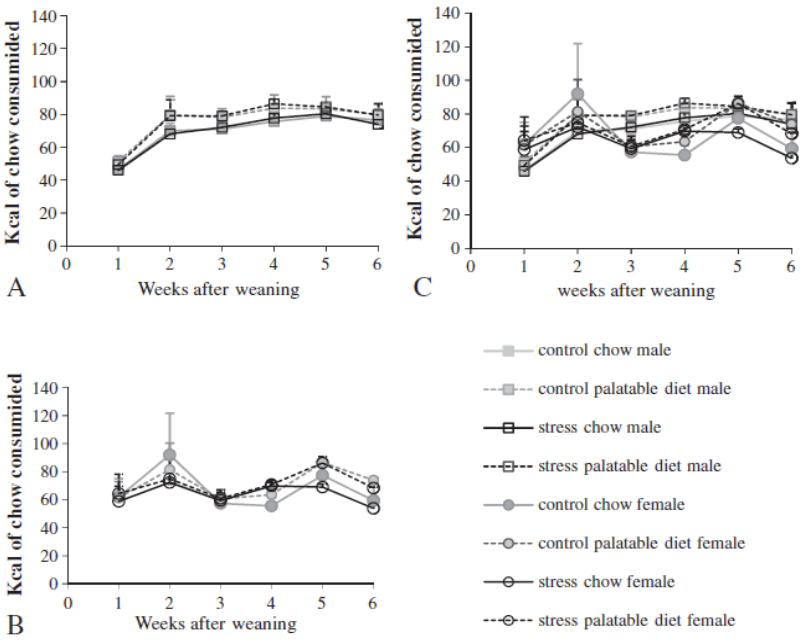

Fig. 3 – Effect of isolation stress during the prepubertal period (PND 21–28) with or without consumption of a palatable diet during the same period on caloric consumption of standard lab chow after PND 28, in male (A) and female (B) rats. Panel (C) shows consumption from all groups for comparison. Data are expressed as mean ± SEM, N = 2–3 cages/group (4–5 animals/cage). After PND 28, animals were reared in groups of 4–5 per cage, receiving standard lab chow. All animals had increased caloric consumption over time (repeated measures ANOVA, $P < 0.001$), with an interaction between time and sex (male rats showed a greater increase in consumption, $P < 0.02$). Animals that had previous access to a palatable diet increased consumption ($P < 0.05$).

the end of the stress exposure. Results show that rats that had previously received palatable diet increased their consumption of regular chow. Exposure to isolation stress during the prepubertal period increased adrenal weight in adult male rats, suggesting that the paradigm of isolation stress used in the early life had an impact on the activation of the HPA axis in adulthood. However, in female rats there was no change in adrenal weight, which may suggest that female rats habituate more easily, or are more able to cope with this type of stress over time.

We also measured the lipid profile in the plasma; exposure to stress increased triacylglycerol levels, and this change remained until adulthood. It is probable that this effect was the result of the increase in caloric consumption. Isolation stress also decreased total cholesterol and LDL-cholesterol levels in adult male rats, while in adult female rats there was an increase in these parameters. Sexual dimorphic effects of stress in relation to lipid metabolism have already been reported [32–34]. Additionally, the sex-specific effects of early stress on cholesterol metabolism did not appear before sexual maturity. It is possible that the higher synthesis of steroid hormones, due to the higher activation of the HPA axis, would lead to a higher use of LDL-cholesterol from the circulation [35].

The adipose tissue releases several adipokines, two of which, leptin and adiponectin, appear to play a role in glucose and lipid metabolism, in energy homeostasis and in the interaction between this last factor and endocrine regulation [36,37]. Isolation stress increased adiponectin in juvenile female rats. Adiponectin is an adipocytokine which has anti-diabetic, anti-atherogenic and anti-inflammatory properties [38,39], and these properties are possibly related to adiponectin's beneficial effects on the cardiovascular system [40]. This increased adiponectin in female rats subjected to isolation stress could be related to the fact that females appear to be more protected against the long-term effects of isolation stress during puberty, when compared to males.

Consumption of caloric dense food and stress exposure, with an excess of glucocorticoids, lead to obesity and increase the risk of developing type 2 diabetes and cardiovascular diseases [41,42], and may disrupt the complex equilibrium of metabolic and neuroendocrine mediators, such as leptin and insulin [43–45]. The palatable diet offered during the prepubertal period decreased plasma adiponectin levels in adult male rats. This adipokine production is reduced in subjects with visceral fat accumulation and its plasma levels are negatively correlated with visceral adiposity [37,46], as was also observed in our study. Hypoadiponectinemia is closely associated with type 2 diabetes, lipid disorders, hypertension and also certain inflammatory diseases [37]. Therefore, its reduced levels may help to explain some of our results, including increased body weight and increased deposition of visceral fat

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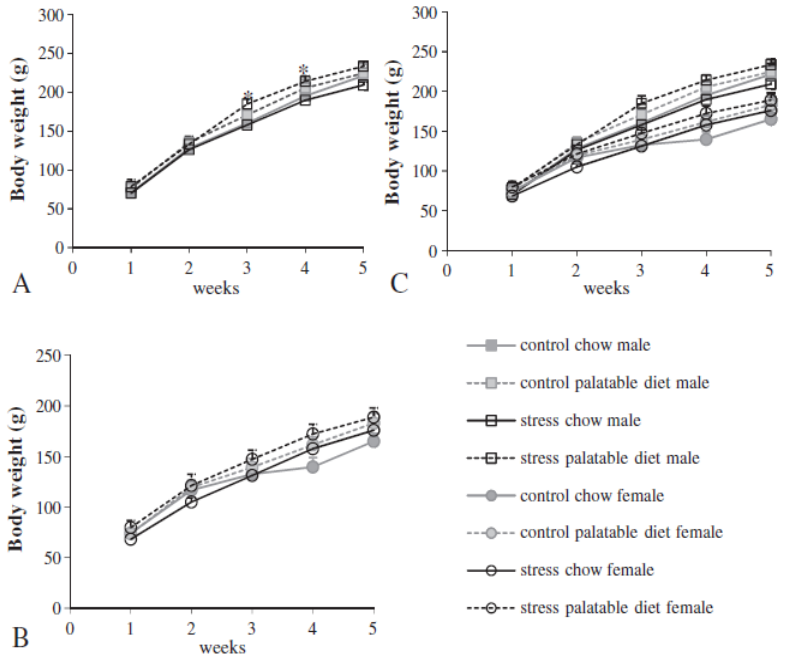

Fig. 4 – Effect of isolation stress during the prepubertal period with or without consumption of a palatable diet during the same period (PND 21–28) on body weight after PND 28, in male (A) and female (B) rats. Panel (C) shows consumption from all groups for comparison. Data are expressed as mean ± SEM, N = 5–10/group. Repeated measures ANOVA showed an increased body weight in males that had received palatable diet during the prepubertal period ($P < 0.05$). No effect was observed between the groups on body weight after PND 28 in female rats ($P > 0.05$). *Different from stressed group receiving standard chow on weeks 3 and 4 (post hoc Duncan test; n = 10 for stress palatable diet group, n = 10 for stress standard chow).

in adult male rats that received the palatable diet during the prepubertal period. Increased glycemia, as well as increased abdominal fat, and reduced adiponectin are considered risk factors for metabolic syndrome [47]. Although these animals do not present metabolic syndrome, they may be at risk of developing this condition.

In this study, the lack of effect of both isolation stress and the palatable food on leptin levels is interesting, since abdominal fat was increased by the palatable diet. This lack of effect could be due to the fact that plasma leptin levels have been reported to correlate mainly with subcutaneous fat [48], while in the present study only visceral fat was measured.

Table 1 – Effects of isolation stress and palatable diet during the prepubertal period on absolute weights of retroperitoneal fat, gonadal fat and relative weights of adrenal glands in adult male and female rats.

Sex		Group			
		Control		Stress	
		Chow	Palatable diet	Chow	Palatable diet
Males	Retroperitoneal fat	2.36 ± 0.24	2.73 ± 0.27 #	1.94 ± 0.12	2.61 ± 0.23 #
	Gonadal fat	2.46 ± 0.16	2.72 ± 0.28	2.39 ± 0.15	2.53 ± 0.16
	Adrenals	0.18 ± 0.013	0.19 ± 0.017	0.21 ± 0.013	0.25 ± 0.034
Females	Retroperitoneal fat	1.83 ± 0.17	1.58 ± 0.18	1.89 ± 0.11	1.68 ± 0.15
	Gonadal fat	2.67 ± 0.37	2.92 ± 0.35	3.54 ± 0.46	3.22 ± 0.38
	Adrenals	0.33 ± 0.021	0.28 ± 0.033	0.28 ± 0.031	0.31 ± 0.034

Fat deposition is shown in grams and adrenal weight is expressed in relation to the body weight of each rat (mg tissue/g of body weight). Data are expressed as mean ± SEM, N = 8–10/group.
Associations between isolation stress and increased adrenal gland weight, and between diet and increased retroperitoneal fat were observed in males (three-way ANOVA, $P < 0.05$).
Different from group exposed to stress, and receiving standard chow (post hoc Duncan test).

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Table 2 – Effects of isolation stress and palatable diet, during the prepubertal period, on plasma triacylglyceride, total cholesterol, LDL-cholesterol and HDL-cholesterol levels in male and female rats at different ages.

Age	Sex		Group			
			Control		Stress	
			Chow	Palatable diet	Chow	Palatable diet
PND-28	Male	Triacylglycerides	36.33 ± 7.47	41.61 ± 10.92	45.41 ± 9.01	47.47 ± 6.56
		Total cholesterol	52.86 ± 5.44	48.67 ± 6.95	49.10 ± 4.05	58.64 ± 7.67
		LDL-cholesterol	21.09 ± 4.87	16.28 ± 5.92	19.37 ± 1.13	28.20 ± 6.86
		HDL-cholesterol	24.50 ± 5.44	24.07 ± 6.83	20.64 ± 1.39	20.94 ± 4.16
	Female	Triacylglycerides	51.31 ± 7.69	44.90 ± 2.76	65.91 ± 7.86	74.70 ± 4.40#
		Total cholesterol	51.83 ± 2.35	43.44 ± 6.23	43.84 ± 9.01	52.14 ± 2.39
		LDL-cholesterol	33.15 ± 2.63	30.80 ± 4.92	18.93 ± 10.23	26.59 ± 3.69
		HDL-cholesterol	9.78 ± 2.83	7.78 ± 0.41	12.02 ± 1.98	10.62 ± 4.73
PND-60	Male	Triacylglycerides	32.41 ± 6.83	30.04 ± 4.18	43.09 ± 5.08	49.51 ± 3.20
		Total cholesterol	45.45 ± 2.48	45.61 ± 0.96	41.71 ± 2.69	38.86 ± 2.78
		LDL-cholesterol	16.89 ± 2.80	18.72 ± 3.71	14.38 ± 5.75	11.37 ± 3.01
		HDL-cholesterol	23.45 ± 3.90	18.33 ± 3.29	18.27 ± 1.25	20.62 ± 3.57
	Female	Triacylglycerides	51.54 ± 6.70	42.83 ± 5.28	56.70 ± 8.43	46.15 ± 6.92
		Total cholesterol	45.56 ± 2.20	49.04 ± 1.29	53.30 ± 3.22	56.13 ± 2.48
		LDL-cholesterol	7.73 ± 2.80	13.30 ± 3.71	14.72 ± 5.75	22.20 ± 3.01
		HDL-cholesterol	24.85 ± 1.42	27.17 ± 2.60	29.35 ± 3.65	24.70 ± 3.50

Data are expressed as mg/dl (mean ± SEM, N = 5/group).
In juvenile rats, there was an effect of stress on triacylglycerol levels; an interaction stress × diet on cholesterol levels resulted from the fact that stress increased total cholesterol levels in animals that received the palatable diet (three-way ANOVA, $P < 0.05$). In adult rats, there was an effect of isolation stress applied during prepuberty, increasing triacylglycerides (three-way ANOVA, $P < 0.05$), and an interaction between stress × sex on total cholesterol and LDL-cholesterol (three-way ANOVA, $P < 0.05$), since exposure to stress increased these parameters in females and decreased them in males.
Different from control groups not exposed to stress (post hoc Duncan test).

The hypothalamus is an important center that controls feeding behavior, and also the stress response. Particularly important in the central regulation of feeding is the orexigenic neuropeptide Y (NPY), which has potent stimulatory effects on food intake in mammals [49,50]. Isolation stress in the prepubertal period induced an increase in the hypothalamic NPY only in juvenile male rats, which could be related to higher stimulation of these neurons, due to marginally higher ghrelin levels in this group, since NPY neurons play a major role in the orexigenic functions of ghrelin [51]. Additionally, the increase in hypothalamic NPY levels after one week of isolation stress may also be ascribed to increased release of

Table 3 – Effects of isolation stress and palatable diet, during the prepubertal period, on plasma glycemia (mg/dl), insulin (ng/mL), adiponectin (μg/mL), leptin (pg/mL) and ghrelin (pg/mL) levels in juvenile male and female rats.

Sex		Group			
		Control		Stress	
		Chow	Palatable diet	Chow	Palatable diet
Male	Glycemia	120.38 ± 3.52	111.30 ± 3.78	100.33 ± 4.31■	112.94 ± 5.66
	Insulin	0.48 ± 0.17	0.01 ± 0.00■	0.11 ± 0.06■	0.03 ± 0.01■
	Adiponectin	14.06 ± 4.08	15.95 ± 1.73	12.98 ± 3.44	13.48 ± 2.76
	Leptin	575.94 ± 118.21	808.95 ± 103.65	754.02 ± 145.35	1241.82 ± 342.18
	Ghrelin	869.70 ± 65.04	994.54 ± 109.49	1818.90 ± 317.03	998.41 ± 30.40
Female	Glycemia	85.53 ± 7.35	88.98 ± 4.48	83.26 ± 2.05	81.02 ± 1.15
	Insulin	0.24 ± 0.10	0.23 ± 0.09	0.06 ± 0.04	0.21 ± 0.10
	Adiponectin	18.62 ± 1.97	12.16 ± 2.54	24.05 ± 2.57★	21.54 ± 2.71★
	Leptin	572.20 ± 105.71	381.72 ± 36.47	530.48 ± 104.75	606.30 ± 789.76
	Ghrelin	1295.51 ± 190.51	903.28 ± 102.52	1399.17 ± 158.97	1799.30 ± 789.76

Data are expressed as mean ± S.E.M., N = 4–5/group.
The palatable diet decreased plasma insulin in males (interaction diet × sex, $P < 0.02$), and an interaction stress × palatable diet × sex was observed for glucose levels (three-way ANOVA, $P = 0.05$). There was an interaction stress × sex, and stress increased plasma adiponectin levels in females (three-way ANOVA, $P < 0.05$).
★ Different from control group receiving palatable diet (Duncan post hoc test).
■ Different from control group receiving standard chow (post hoc Duncan test).

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Table 4 – Effects of isolation stress and palatable diet, during the prepubertal period, on plasma glycemia (mg/dl), insulin (ng/mL), adiponectin (μg/mL), leptin (pg/mL) and ghrelin (pg/mL) levels in adult male and female rats.

Sex		Group			
		Control		Stress	
		Chow	Palatable diet	Chow	Palatable diet
Male	Glycemia	74.86 ± 1.27	77.29 ± 1.66	70.23 ± 3.92	79.18 ± 2.52
	Insulin	0.91 ± 0.14	0.53 ± 0.06	0.87 ± 0.10	0.90 ± 0.17
	Adiponectin	18.92 ± 3.42	10.18 ± 0.52	20.81 ± 3.59	12.24 ± 3.24 ♦
	Leptin	357.98 ± 75.79	467.11 ± 119.47	431.53 ± 96.83	511.19 ± 127.54
	Ghrelin	1346.95 ± 349.17	994.54 ± 152.58	903.28 ± 108.54	1762.95 ± 613.30
Female	Glycemia	117.41 ± 4.47	100.98 ± 8.00 *	105.53 ± 3.76	109.39 ± 1.83
	Insulin	0.59 ± 0.12	0.76 ± 0.26	0.41 ± 0.07	0.99 ± 0.39
	Adiponectin	19.47 ± 1.04	19.82 ± 3.62	22.39 ± 2.31	18.46 ± 2.70
	Leptin	516.53 ± 48.81	640.47 ± 145.06	514.29 ± 25.66	481.63 ± 72.27
	Ghrelin	2014.22 ± 248.84	1777.18 ± 399.45	3139.38 ± 1485.72	1407.39 ± 149.50

Data are expressed as mean ± SEM, N = 4-5/group.
There were interactions stress × palatable diet (three-way ANOVA, $P < 0.001$), and palatable diet × sex ($P < 0.05$) on glycemia. An effect of sex ($P < 0.05$), as well as an effect of exposure to palatable diet ($P < 0.02$) was observed on adiponectin levels.
* Different from control group receiving standard chow (post hoc Duncan test).
♦ Different from respective control group (post hoc Duncan test).

glucocorticoids, which can act on the central nervous system, modulating food intake, possibly through activation of NPY [15,52,53]. It is possible that the increase in the hypothalamic NPY and ghrelin levels, in juvenile male rats, induced by stress in the prepubertal period may be related to the increase in caloric consumption during the same period, considering the involvement of these peptides in food intake. Furthermore, sex-specific differences were notable, since the juvenile female rats did not demonstrate changes in the levels of these peptides.

In conclusion, isolation stress and consumption of palatable diet in the prepubertal period can change metabolism at different stages of life in a sex-specific manner. Prepubertal female rats were more prone to stress effects on food consumption during this period; they are also more susceptible than male rats to the use of a palatable diet as comfort food, and future translation of these results to humans could help to understand why women are more susceptible to feeding disorders. Male rats, on the other hand, demonstrated more long-lasting effects of these interventions, being more susceptible to a metabolic programming after consumption of a palatable diet during these early periods. It is important to point out that, for some of the measurements, the sample size was small; therefore, it is possible that some small effects were not detected and some caution must be taken when interpreting these results.

These findings point to stress and diet as causal factors for metabolic programming during the prepubertal period. Additionally, these sex differences related to the response to stress in the prepubertal period may have implications on the vulnerability to stress-related disorders in the adulthood. We believe that the research concerning early interventions will enable us to understand how these modifications occur, and the identification of environmental factors that may increase vulnerability will enable us to prevent chronic conditions in the adult. Therefore, these results may contribute to the enlightenment of sex differences concerning future therapeutic and preventive approaches on the effects of stressors early in life.

Table 5 – Effects of isolation stress and palatable diet, during the prepubertal period, on hypothalamic NPY (ng/mg prot) in male and female rats at different ages.

Age	Sex	Control		Stress	
		Chow	Palatable diet	Chow	Palatable diet
PND-28	Male	6.76 ± 1	11.97 ± 4.01	38.23 ± 12.52 #	24.35 ± 9.26
	Female	0.83 ± 0.02	0.91 ± 0.07	0.92 ± 0.06	0.80 ± 0.08
PND-60	Male	1.24 ± 0.25	0.81 ± 0.16	1.13 ± 0.15	0.55 ± 0.09 *
	Female	0.67 ± 0.02	0.67 ± 0.03	0.68 ± 0.05	0.77 ± 0.05

Data are expressed as mean ± SEM, N = 4–9/group.
There was an effect of isolation stress in juvenile male rats (three-way ANOVA, interaction stress × sex; $P < 0.05$), which increased hypothalamic NPY. In adult male rats there was an effect of palatable diet, which decreased hypothalamic NPY (interaction diet × sex; $P < 0.05$).
Different from groups not exposed to stress (post hoc Duncan test).
* Different from control group receiving standard chow (post hoc Duncan test).

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Authors' contributions

Krolow R participated in the implementation and supervision of all experiments; Noschang C, Arcego DM, Marcolin ML, and Huffel AP participated in developing the methodology, especially with the accompanying the isolation stress and consumption of diet palatable; Noschang C, Lampert C, Benitz AN, and Fitarelli RD, participated in biochemical experiments. Dalmaz C directed and oversaw all development of this work.

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Conflict of interest

This paper does not present a conflict of interest.

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CAPÍTULO 2

Manuscrito: Isolation stress during the prepubertal period in rats induces long-lasting neurochemical changes in the prefrontal córtex.

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ORIGINAL PAPER

Isolation Stress During the Prepubertal Period in Rats Induces Long-Lasting Neurochemical Changes in the Prefrontal Cortex

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Abstract Social isolation during postnatal development leads to behavioral and neurochemical changes, and a particular susceptibility of the prefrontal cortex to interventions during this period has been suggested. In addition, some studies showed that consumption of a palatable diet reduces some of the stress effects. Therefore, our aim is to investigate the effect of isolation stress in early life on some parameters of oxidative stress and energy metabolism (Na^+,K^+-ATPase activity, respiratory chain enzymes activities and mitochondrial mass and potential) in prefrontal cortex of juvenile and adult male rats. We also verified if the consumption of a palatable diet during the prepubertal period would reduce stress effects. The results showed that, in juvenile animals, isolation stress increased superoxide dismutase and Complex IV activities and these effects were still observed in the adulthood. An interaction between stress and diet was observed in catalase activity in juveniles, while only the stress effect was detected in adults, reducing catalase activity. Access to a palatable diet increased Na^+,K^+-ATPase activity in juveniles, an effect that was reversed after removing this diet. On the other hand, isolation stress induced a decreased activity of this enzyme in adulthood. No effects were observed on glutathione peroxidase, total thiols and free radicals production, as well as on mitochondrial mass and potential. In conclusion, isolation stress in the prepubertal period leads to long-lasting changes on antioxidant enzymes and energetic metabolism in the prefrontal cortex of male rats, and a palatable diet was not able to reverse these stress-induced effects.

Keywords Pre-puberty · High palatable diet · Isolation stress · Oxidative stress · Respiratory chain · Mitochondrial mass and potential

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Introduction

The stress response involves the neuroendocrine activation of sympatho-adrenomedullar system, release of catecholamines, and activation of hypothalamic–pituitary–adrenal (HPA) axis, culminating in the release of glucocorticoids (GCs) [1]. Epidemiologic studies, as well as studies using animal models, determined that early adverse experiences may lead to abnormal behavior associated with alterations of the HPA axis [2, 3]. In rats, during puberty this axis functions in a different way compared with adults, for example, the corticosterone secretion after exposure to an acute stressor is delayed but more prolonged when compared to adult rats, and habituation to stressors is less efficient [4].

Social isolation, considered a type of psychological stress in rats [5–7], can lead to behavioral, anatomical and neurochemical changes when applied during early postnatal development, and these changes may also be present in adulthood, when these animals are compared to their socially reared litter mates (socials) [2, 8]. The peripuberal period in rats is a time of transition, sexual maturation, and enhanced brain architecture plasticity. In addition, this period is critical for the final maturation of circuits controlling energy homeostasis and stress responses [9], and exposure to certain situations, such as stressors, in this

phase of life may influence diseases susceptibility or resilience in adulthood [10]. The prefrontal cortex, considered a late maturing brain structure, is particularly susceptible to interventions during puberty [3].

Glucocorticoids, released during the stress response, have been associated with increased palatable food intake [11]. The increasing stress of daily life has been associated with an increased motivation for foods rich in lipids and carbohydrates [12, 13] and a model of reward-based eating has been suggested as a mean to reduce the stress response [14]. In this sense, the possibility of an interaction between the effects of stress and consumption of easily available palatable food has been suggested [15].

The adaptive response to stress involves important changes in mitochondrial functions, enabling them to adjust bioenergetics, thermogenesis, oxidative and/or apoptotic responses [16]. In addition, elevated levels of GCs may lead to an increased generation of reactive oxygen species (ROS) and the imbalance between high cellular levels of ROS in relation to cellular antioxidant defenses, namely oxidative stress, may be involved in the pathogenesis of several brain diseases [17–21]. ROS can induce mitochondrial dysfunction, disruption of energy pathways [22], damage to neuronal precursors and impairments in neurogenesis [23]. In addition, Na^+,K^+-ATPase, which is an enzyme responsible for maintaining the electrochemical gradient necessary for neuronal excitability and regulation of neuronal cell volume, is susceptible to free radicals attack [24], and studies using animal models have reported decreased activity of Na^+,K^+-ATPase after chronic mild stress [25].

Therefore, the aim of this study is to investigate the effect of isolation stress in early life on some parameters of oxidative stress and energy metabolism (Na^+,K^+-ATPase activity, respiratory chain enzymes activities and mitochondrial mass, as well as the potential) in medial prefrontal cortex of juvenile (28 days of age) and adult (60 days of age) male rats. Our hypothesis was that isolation stress in early life can lead to long-term mitochondrial dysfunction in the prefrontal cortex. We also verified if the consumption of a palatable diet would reduce stress effects during the prepubertal period.

Materials and Methods

Subjects

All animal proceedings were approved by the Institutional Ethical Committee and followed the recommendations of the International Council for Laboratory Animal Science (ICLAS) and of the Federation of Brazilian Societies for Experimental Biology. All efforts were done to minimize animal suffering as well as to reduce the number of animals.

Forty Wistar rats, from 10 litter, were weaned on postnatal day 21. A total of four male pups were used from each litter, and these four pups were divided into four groups, as described below, in such a way that one animal per litter was used in each group. Male pups were weighed and distributed into four groups, receiving a high palatable diet [26] or standard lab chow. Half of the animals were housed in groups of 5; the other half were stressed by isolation (one animal in a smaller home cage, 27 × 17 × 12 cm) [27], in such a way that four groups resulted: controls receiving standard lab chow, controls receiving palatable diet, isolated receiving lab chow, and isolated receiving palatable diet. These interventions occurred between postnatal days 21–28 and daily food consumption was measured. At postnatal day 28, half of the animals were killed by decapitation and biochemical evaluations were performed, measuring the activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), total thiols, reactive species (through oxidation of dichlorodihydrofluorescein, DCFH), Na^+,K^+-ATPase activity, respiratory chain complexes activity, mitochondrial mass and potential ($\Delta\psi$) (using Mito Tracker). The other half of the animals received standard chow and was reared in groups of 5 per cage until adulthood when they were killed by decapitation and the same biochemical parameters were assessed.

Palatable Diet

The high palatable diet used on this study is enriched in simple carbohydrates, and it is made with condensed milk, sucrose, vitamins and salts mix, powder standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet is similar to that of a standard lab chow (including 22% protein and 4–6% fat), however, most carbohydrates in the palatable diet were sucrose (from condensed milk and from sucrose); in contrast, the standard lab chow had carbohydrates mainly from starch.

Assessment of Oxidative Stress Parameters

The animals were killed by decapitation. Their medial prefrontal cortex (including anterior cingulate, infralimbic and prelimbic regions) was quickly dissected out and stored at −70°C until analysis, when the structures were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate was centrifuged at 1,000×*g* for 10 min at 4°C and the supernatant was used.

Superoxide Dismutase Activity

Superoxide dismutase activity was determined using a RANSOD kit (Randox Labs., USA) which is based on the

procedure described by Delmas-Beauvieux et al. [28]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed spectrophotometrically at 492 nm at 37°C. The inhibition in the production of the chromogen is proportional to the activity of SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Catalase Activity

Catalase activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25°C [29]. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined according to Wendel [30] with modifications. The reaction was carried out at 37°C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione, and 0.4 U glutathione reductase. The activity of GPx was measured taking tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg protein.

Evaluation of Free Radicals Production by the Chemical Oxidation of Dichlorodihydrofluorescein (DCFH)

The samples were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (100 μM) at 37°C for 30 min. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored by excitation and emission wavelength of 488 and 525 nm, respectively, using a spectrum photometer. The formation of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmol of DCF formed per mg of protein [31].

Determination of Total Thiol

This essay is based in the reduction of 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) by thiol groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein [32].

Determination of Na^+,K^+-ATPase Activity

For Na^+,K^+-ATPase activity determination, prefrontal cortex was homogenized in 10 vol (w:v) of 0.32 M sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4 [33]. The homogenates were centrifuged at 1,000×*g* for 10 min and the supernatants were used. The reaction mixture contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl and 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μL. The reaction started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3 mM. Controls were assayed under the same conditions with the addition of 1 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays as described by Wyse et al. [33]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [34]; enzyme specific activity was expressed as nmol Pi released per minute per mg protein.

Respiratory Chain Activity Determination

Mitochondrial energy metabolism was evaluated using enzymatic analysis of the electron transport chain (ETC.) activities. For determination of respiratory chain complexes activities, brain structures were homogenized with a teflon–glass homogenizer (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base), pH 7.4. The homogenates were centrifuged at 1,000×*g* for 10 min and the supernatants were immediately kept at −70°C until used for enzyme activity determination. The activities of the ETC. complexes I–III, II and IV were determined in homogenates according to standard methods previously described in the literature [35–37]. The activity of complex I–III (complex I + CoQ + III) was assessed by measuring the increase in absorbance due to cytochrome c reduction at 550 nm according to the method described by Schapira et al. [37]. The reaction mixture contained 8–15 μg protein and 20 mM potassium phosphate buffer with 2 mM KCN, 10 mM EDTA and 50 mM cytochrome c, pH 8.0. The reaction was initiated adding 25 mM NADH and was monitored at 25°C for 3 min before addition of 10 mM rotenone, after which the activity was measured for an additional 3 min. Complex I–III activity was the rotenone sensitive NADH: cytochrome c reductase activity. The activity of complex II (succinate: DCIP oxireductase) was determined according to Fischer et al. [35], following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm. After a pre-incubation with 30–60 μg protein for 20 min, the reaction was carried out at 30°C in a medium consisting of 40 mM potassium phosphate buffer

containing 16.0 mM sodium succinate and 8 mM DCIP, pH 7.4. After that, 4 mM sodium azide, 7 mM rotenone and 40 mM DCIP were added to the medium and monitored for 5 min. Cytochrome c oxidase (COX, complex IV) activity was determined according to Rustin et al. [36], following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm. The reaction was initiated adding 0.7 mg reduced cytochrome c in a medium containing 10 mM potassium phosphate buffer, 0.6 mM *n*-dodecyl-*β*-D-maltoside, pH 7.0 and 1.5–3 μg protein. The activity of complex IV was measured at 25°C for 10 min. The activity of respiratory chain complexes were calculated and expressed as nmol per min per mg protein.

Mitochondrial Mass and Membrane Potential Measurement

Mitotracker was used for mitochondrial function analysis in cell suspensions of cerebral cortex obtained by mechanical dissociation with PBS containing collagenase to favor digestion to a density of about 200,000 cells/mL. The dissociated contents were then filtered into a sterile 50 mL Falcon tubes (BD Biosciences) through 40 μm nylon cell strainer (Cell Filter Strainer—BD Biosciences) and kept on ice until mitochondrial staining. To assess mitochondrial function, MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG) dyes were employed. MTR is a lipophilic cationic fluorescent dye that is concentrated inside mitochondria by their negative mitochondrial membrane potential [38]. The loss of membrane potential as mitochondria depolarized results in release of MTR from the mitochondria and a subsequent decrease in fluorescence [39]. MTG is a green-fluorescing fluorophore that is taken up electrophoretically into polarized mitochondria and has been used as a measure of mitochondrial mass independent of mitochondrial membrane potential. Chloromethyl groups on MTG form covalent adducts with sulfhydryls of mitochondrial matrix proteins such that MTG is retained even after mitochondrial depolarization [40]. MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock concentration. Dissociated tissue content, previously filtered, was stained with 100 nM MTR and 100 nM MTG for 45 min at 37°C in a water bath in a dark room, according to the method described by Keij et al. [41] and Pendergrass et al. [38], with some modifications. Immediately after staining cell suspensions were removed from the water bath and analyzed by flow cytometry.

For Flow Cytometry Analysis, samples stained with MTR and MTG dyes were analyzed on the FACSCalibur (Becton–Dickinson, San Jose, CA). MitoTracker dyes were excited using 488 air-cooled argon laser. Negative controls (samples without stain) were included for setting up the machine voltages. Controls stained with a single dye were also employed to allow the setting of compensation. The emission of fluorochromes was recorded through specific band-pass fluorescence filters: red (FL-3; 670 nm long pass) and green (FL-1; 530 nm/30). Fluorescence emissions were collected using logarithmic amplification. Briefly, data from 10,000 events (intact cells) were acquired and mean relative fluorescence intensity was determined after exclusion of debris events from the listmode data set. All flow cytometric acquisitions and analyses were performed using CELLQuest Pro data acquisition (BD Biosciences) and FlowJo analysis software. Data were analyzed and plotted by density as a single-parameter histogram that shows the relative fluorescence on the *x*-axis and the number of events (cell count) on the *y*-axis. Flow cytometry analyzed samples produced a single distinct peak that can be interpreted as the positive dataset. The histograms were divided in two halves (named cells with low and high mass or potential), based on the peak of the controls for MTR and MTG, and this evaluation were extended to all data in each parameter. Using this method, analyses of cells resulted in two populations, with different mitochondrial $\Delta\psi$. The first population presented low $\Delta\psi$ and the second, high $\Delta\psi$ (the same procedure was used for low and high mitochondrial mass). The lower accumulation of MTR or MTG, and thus lower fluorescence values would be indicative of decreased mitochondrial mass or $\Delta\psi$ [42, 43].

Protein Assay

The total protein concentration was determined using the method described by Lowry et al. [44] with bovine serum albumin as the standard. For determination of Na^+,K^+-ATPase activity, total protein concentration was measured by Bradford [45].

Statistical Analysis

Data were expressed as mean ± SE of the mean, and were analyzed using two-way ANOVA, with isolation stress and palatable diet as factors.

Results

Antioxidant Enzymes Activities, Total Thiol Level and Free Radicals Production

These parameters were analyzed to verify if there was an oxidative imbalance in prefrontal cortex after exposure to isolation stress in the prepuberty period. These measurements were performed at 28 days (juveniles) and at 60 days

of age (adults). Figure 1 shows the results for juvenile animals. Both isolation stress [$F(1, 16) = 16.73, P < 0.01$] and palatable diet [$F$ (1, 16) = 6.78, $P < 0.05$] increased SOD activity. An interaction between stress and palatable diet [$F(1, 16) = 8.13, P < 0.05$] was observed when evaluating CAT activity, since a potentiation occurred in its activity when both factors were present. The increase induced by isolation stress on SOD activity was long-lasting, since it was still observed in adulthood [F (1, 16) = 428.19, $P < 0.01$] (Fig. 2), when a decreased CAT activity was also observed in rats exposed to isolation in the prepubertal period [F(1, 16) = 2.70, $P < 0.05$]. GPX activity was not altered by isolation stress or by access to a palatable diet in any of the ages analyzed ($P > 0.05$).

SOD/GPx ratio (Table 1) was increased by isolation stress at 28 days of age (juveniles) [F (1, 15) = 0,054, $P < 0.01$] in prefrontal cortex, while there was no difference in the SOD/CAT ratio (Table 2). At 60 days of age (adults), stress increased both SOD/GPx [$F(1, 16) = 0.55, P < 0.01$] and SOD/CAT [$F$ (1, 16) = 401.85, $P < 0.01$] ratios.

There was no effect on total thiol levels or free radicals production as evaluated through the DCF test, neither in juveniles nor in adult animals ($P > 0.05$).

Na^+,K^+-ATPase activity

The access to a palatable diet during the prepubertal period increased Na^+,K^+-ATPase activity [F (1, 13) = 899.97, $P < 0.01$] in the prefrontal cortex of juveniles (Fig. 3). When the animals returned to a standard chow until adulthood, this effect of the diet was reversed (Fig. 4); additionally, adult animals subjected to isolation stress in the prepubertal period presented a decrease in Na^+,K^+-ATPase activity [F (1, 18) = 339.61, $P < 0.05$].

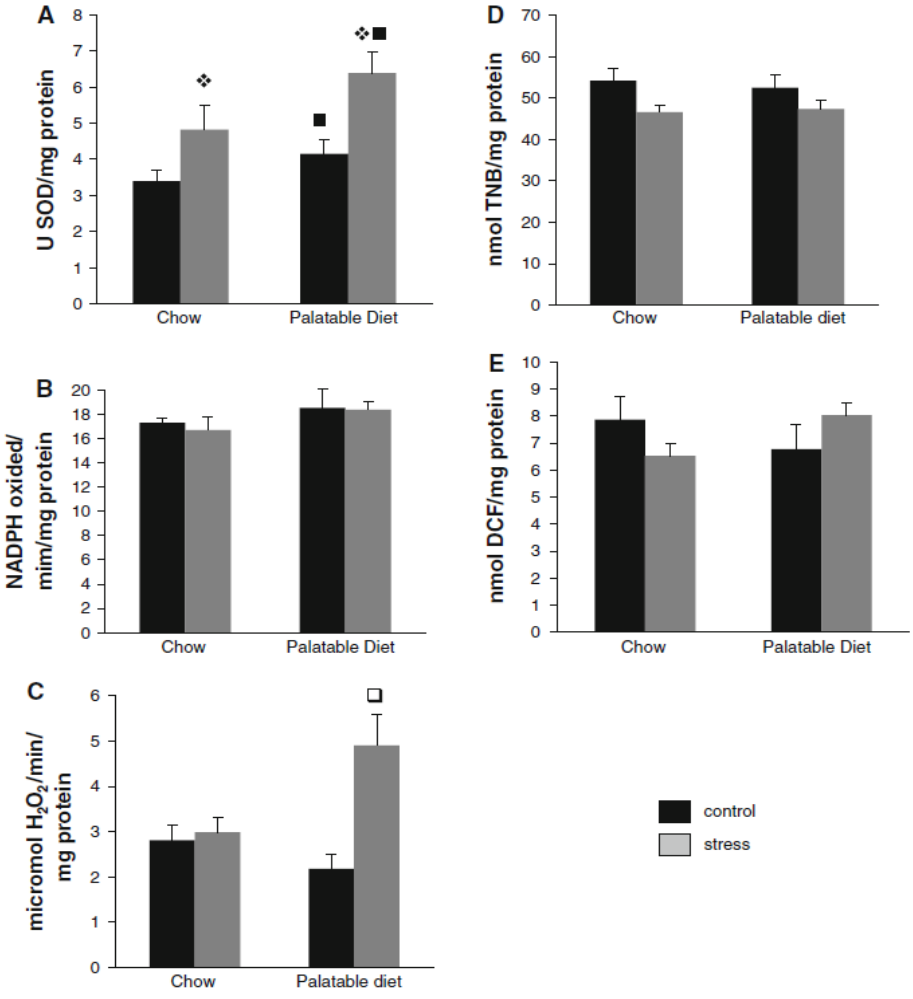

Fig. 1 Effects of isolation stress and consumption of palatable diet on antioxidant enzymes activities, total thiol and free radicals (DCFH test) production in the prefrontal cortex of juvenile animals. Data are expressed as mean ± SEM. $N = 4$–5/group. **a** SOD (expressed as U/mg protein), **b** GPx (expressed as nmol NADPH oxidized/min/mg protein), **c** CAT (expressed as micromoles of H_2O_2 consumed/ min/mg protein), **d** total Thiol (expressed as nmol TNB/mg protein) and **e** DCFH (expressed as nmol of DCF formed/mg protein). *Asterisk* Main effect of isolation stress and *filled square* palatable diet, both increasing SOD activity ($P < 0.05$) (two-way ANOVA, $P < 0.05$). *Square* Significant interaction between stress and palatable diet on CAT activity (two-way ANOVA, $P < 0.05$)

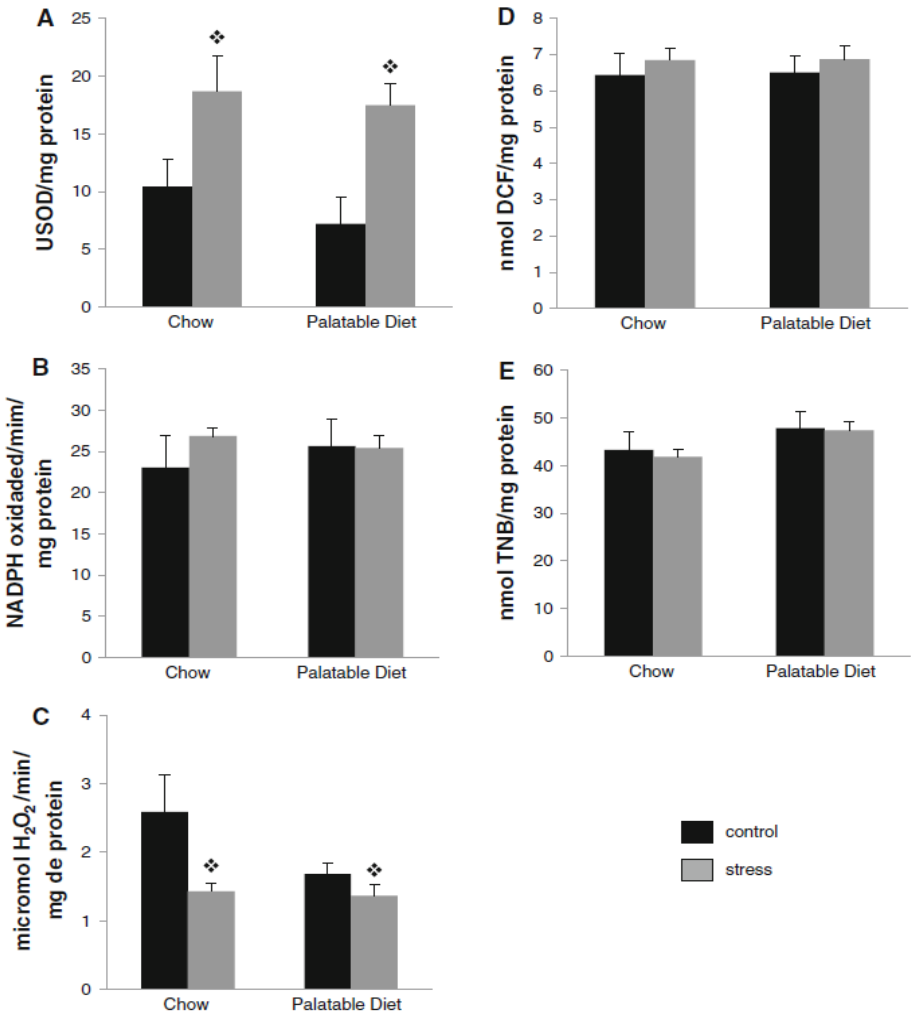

Fig. 2 Effects of isolation stress and consumption of palatable diet on antioxidant enzymes activities, total Thiol and free radicals (DCFH test) production in the prefrontal cortex of adult rats. Data are expressed as mean ± SEM $N = 4$–5/group. **a** SOD (expressed as U/mg protein), **b** GPx (expressed as nmol NADPH oxidized/min/mg protein), **c** CAT (expressed as micromoles of H_2O_2 consumed/ min/mg protein), **d** total Thiol (expressed as nmol TNB/mg protein) and **e** DCFH (expressed as nmol of DCF formed/mg protein). *Asterisk* Main effect of isolation stress increasing SOD activity ($P < 0.01$) and decreasing CAT activity ($P < 0.05$) (two-way ANOVA)

Table 1 Effects of isolation stress and consumption of palatable diet during the prepubertal period on SOD/GPx ratio in the prefrontal cortex of juvenile and adult rats

Group	Juveniles	Adults
Control + chow	0.19 ± 0.02 (5)	0.46 ± 0.07 (5)
Control + palatable diet	0.23 ± 0.02 (5)	0.28 ± 0.07 (5)
Stressed + chow	0.30 ± 0.05 (5)*	0.69 ± 0.10 (5)*
Stressed + palatable diet	0.35 ± 0.02 (5)*	0.70 ± 0.09 (4)*

Data are expressed as mean ± SEM (n)

* Main effect of isolation stress (two-way ANOVA, $P < 0.01$) both at juvenile and adult groups (28 and at 60 days of age)

Table 2 Effects of isolation stress and consumption of palatable diet during the prepubertal period on SOD/CAT ratio in the prefrontal cortex at juvenile and adult animals

Group	Juveniles	Adults
Control + chow	1.30 ± 0.22	4.72 ± 1.20
Control + palatable diet	2.11 ± 0.40	4.70 ± 1.84
Stressed + chow	1.69 ± 0.31	13.83 ± 3.20*
Stressed + palatable diet	1.42 ± 0.26	13.51 ± 2.38*

Data are expressed as mean ± SEM

* Main effect of isolation stress at 60 days of age (two-way ANOVA, $P < 0.01$). $N = 5$/group

Respiratory Chain Enzymes Activities

Mitochondrial energy metabolism in the prefrontal cortex was evaluated using enzymatic analysis of (ETC) activities.

Isolation stress during the prepubertal period increased complex IV activity in juveniles [$F(1, 16) = 939.82$, $P < 0.05$] (Fig. 5), and this increase remained until

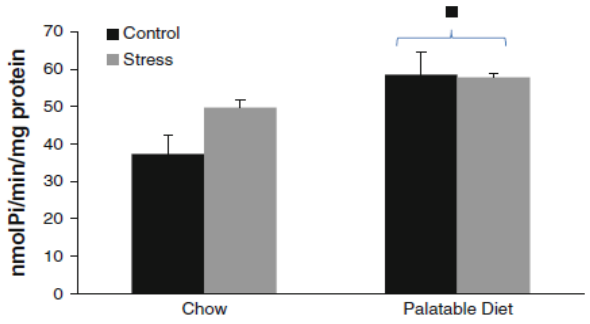

Fig. 3 Effects of isolation stress and consumption of palatable diet during the prepubertal period on Na^+,K^+-ATPase activity (expressed as nmol Pi released/min/mg protein) in the prefrontal cortex of juveniles (28 days of age). Data are expressed as mean ± SEM. $N = 4$–6/group. *Filled square* Main effect of the palatable diet, increasing Na^+,K^+-ATPase activity (two-way ANOVA, $P < 0.01$)

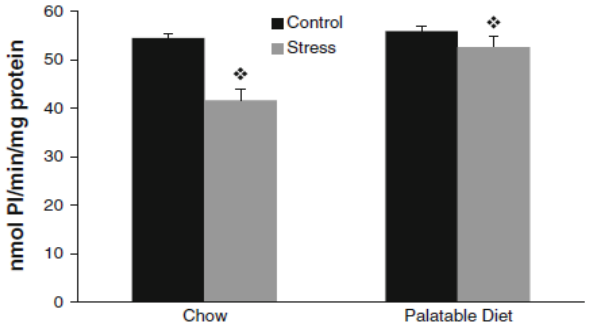

Fig. 4 Effect of isolation stress and consumption of palatable diet during the prepubertal period on Na^+,K^+-ATPase activity (expressed as nmol Pi released/min/mg protein) in the prefrontal cortex of adult rats. Data are expressed as mean ± SEM. $N = 5$–6/group. *Asterisk* Main effect of stress, decreasing Na^+,K^+-ATPase activity (two-way Anova, $P < 0.05$)

adulthood [$F(1, 11) = 9{,}756.51$, $P < 0.01$] (Fig. 6). In addition, the palatable diet decreased Complex IV activity [$F(1, 11) = 4{,}497.34$, $P < 0.05$], but only in adulthood. Enzymes activities for Complex I + III and Complex II were not significantly different ($P > 0.05$).

Mitochondrial Mass and Membrane Potential

Analysis of cerebral prefrontal cortex cells labeled with MTG and MTR are shown in Tables 3 and 4, respectively. There was no significant difference neither in mitochondrial mass nor in mitochondrial $\Delta\psi$ ($P > 0.05$).

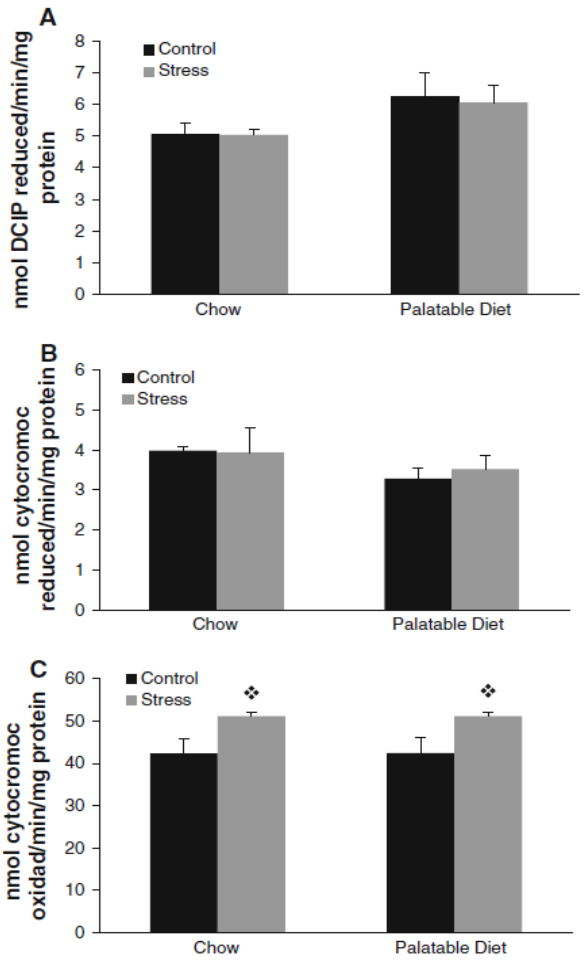

Fig. 5 Effects of isolation stress and consumption of palatable diet during the prepubertal period on respiratory chain enzymes activities in the prefrontal cortex of juvenile animals. Data are expressed as mean ± SEM $N = 4$–5/group. **a** complex II activity (nmol DCIP reduced/min/mg protein), **b** complex I–III activity (nmol cytocromo c reduced/min/mg protein) and **c** Complex IV activity (nmol cytocromo c oxidized/min/mg protein). *Asterisk* Main effect of stress, increasing complex IV activity (two-way ANOVA, $P < 0.05$)

Discussion

The results of this work showed that neurochemical parameters such as activities of antioxidant enzymes, enzymes of respiratory chain, and Na^+,K^+-ATPase were altered in prefrontal cortex of rats subjected to isolation stress in prepubertal period; these effects were not prevented by palatable diet. In the juvenile rats, the results showed that isolation stress and palatable diet increased SOD activity, and stress increased SOD/GPx ratio, while both factors potentiate each other increasing CAT activity.

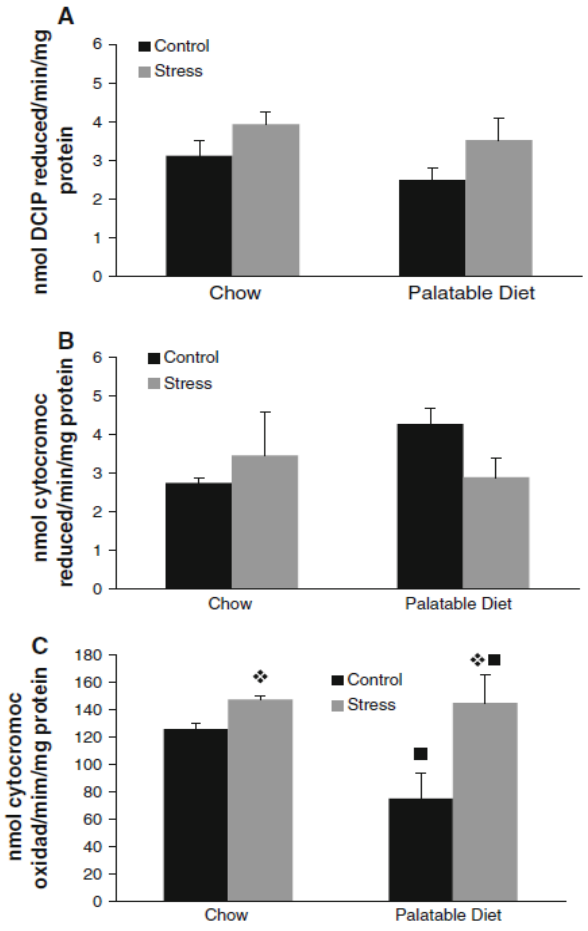

Fig. 6 Effects of isolation stress and consumption of palatable diet during the prepubertal period on respiratory chain enzymes activities in the prefrontal cortex of adult rats. Data are expressed as mean ± SEM $N = 4$–5/group. **a** Complex II activity (nmol DCIP reduced/min/mg protein), **b** complex I–III activity (nmol cytocromo c reduced/min/mg protein) and **c** complex IV activity (nmol cytocromo c oxidized/min/mg protein). *Asterisk* Main effects of stress increasing ($P < 0.01$) and *filled square* palatable diet decreasing complex IV activity ($P < 0.05$) (two-way ANOVA)

However, in the adult rats, stress increased SOD activity, decreased CAT activity, increased SOD/GPx and SOD/CAT ratios. These results suggest that the changes induced by isolation stress on antioxidant enzymes activities were long term effects. The increased ratios, due to increased SOD activity, may result on higher concentration of H_2O_2, and these ratios have been used as an indication of peroxide overload challenge [46, 47]. The imbalance that included increased SOD activity, leading to increased hydrogen peroxide, but no change (juveniles) or reduction (CAT, in adults) in the enzymatic antioxidant systems (CAT and GPx) responsible for neutralizing the hydrogen peroxide could lead to an excess of H_2O_2, facilitating the production of hydroxyl radical ($OH^{\bullet}$), the most powerful oxidant molecule, through a reaction with iron or copper (Fenton chemistry) [48]. Agreeing with our results, H_2O_2 production has been reported to be enhanced after stress [49]. Another type of psychological stress, the restraint stress, has also been reported to result in the imbalance of antioxidant status, which ultimately leads to induction of oxidative damage [50, 51]. Our study points to long term effects of stress on oxidative status, which last during weeks after stress exposure is interrupted.

Although access to a palatable diet increased SOD activity at 28 days of age, this increase did not remain until adulthood, indicating that this antioxidant enzyme returned to normal when the palatable diet was withdraw. Some studies show that administration of high fat or high caloric diets to rodents increases free radical generation in the brain [52]. A previous study from our laboratory showed that animals' chronically receiving chocolate in adulthood had altered activity of antioxidant enzymes [53]. In the present study, however, the palatable diet was administered for a week in prepubertal period. It is interesting to observe that, although access to a palatable diet has been suggested to counteract some of the effects induced by stress exposure [15], the only interaction observed between diet and stress exposure was on CAT activity, and this interaction (potentiating this enzyme activity) was not able to significantly influence SOD/CAT ratio.

Our results showed that the palatable diet increased Na^+,K^+-ATPase activity in juvenile rats while isolation stress during the prepubertal period decreased Na^+,K^+-ATPase activity in adult rats. Chronic stress is known to decrease Na^+,K^+-ATPase activity in adulthood [54, 55], an effect that is reversed when stress is interrupted [54]. In the present study, exposure to stress during development led to effects later in life, decreasing Na^+,K^+-ATPase activity in the prefrontal cortex. This reduction in Na^+,K^+-ATPase activity may compromise neurotransmission, since this enzyme is mainly responsible for the generation of the membrane potential necessary to maintain neuronal excitability [56]. We can not attribute the inhibition of Na^+,K^+-ATPase activity to reduced energy production, at least considering the present results, since stress increased the activity of Complex IV, having no effect on other complexes of the respiratory chain. This decreased Na^+,K^+-ATPase activity may be related to the altered oxidative status induced by isolation stress, which remains altered several weeks after interruption of stress exposure (until adulthood), since this enzyme is susceptible to free radicals attack [57], and to reduced antioxidants or antioxidant enzymes activities [58].

Regarding the effect of consumption of a palatable diet on Na^+,K^+-ATPase activity, it disappeared after

Table 3 Effects of isolation stress and consumption of palatable diet during the prepubertal period on mitochondrial mass in the prefrontal cortex in juveniles and adults

	Group	Juveniles	Adults
Low mass	Control + chow	4,302.2 ± 50.6	4,859.8 ± 5,992.7
	Control + palatable diet	4,117.8 ± 218.0	5,375.6 ± 303.4
	Stressed + chow	4,150.6 ± 164.3	5,232.4 ± 485.0
	Stressed + palatable diet	4,192.8 ± 192.8	5,877.2 ± 716.2
High mass	Control + chow	3,719.8 ± 212.0	4,856.2 ± 625.4
	Control + palatable diet	4,195.6 ± 494.4	4,298.4 ± 318.1
	Stressed + chow	4,276.2 ± 381.8	4,178.8 ± 521.9
	Stressed + palatable diet	4,015.0 ± 472.0	3,727.4 ± 765.0

Data are expressed as mean ± SEM

No significant effects were observed ($P > 0.05$). $N = 4$–5/group

Table 4 Effects of isolation stress and consumption of palatable diet during the prepubertal period on mitochondrial potential in the prefrontal cortex in juvenile and adult animals

	Group	Juveniles	Adults
Low potential	Control + chow	4,127.8 ± 145.1	4,856.2 ± 318.4
	Control + palatable diet	4,076.4 ± 252.6	5,138.6 ± 124.6
	Stressed + chow	3,999.8 ± 324.6	4,728.2 ± 292.8
	Stressed + palatable diet	4,408.8 ± 176.1	5,176.0 ± 358.8
High potential	Control + chow	4,149.8 ± 438.6	4,037.6 ± 517.2
	Control + palatable diet	4,312.6 ± 553.2	3,586 ± 201.4
	Stressed + chow	4,499.2 ± 585.9	3,577.2 ± 590.0
	Stressed + palatable diet	3,817.2 ± 436.9	3,237.2 ± 570.9

Data are expressed as mean ± SEM

No significant effects were observed ($P > 0.05$). $N = 4$–5/group

withdrawal of this diet, when animals received regular rat chow. In contrast, previous studies showed that exposure to palatable diets may reduce Na^+,K^+-ATPase activity in hippocampus and amygdale [59]. It may be important to point out that our study differs from these other ones in which Na^+,K^+-ATPase activity was evaluated because we studied the cerebral cortex during development, a period in which rapid changes in synaptic density are occurring [60, 61]. Interestingly, diet-induced changes in Na^+,K^+-ATPase activity were reversed when the diet was discontinued, as was observed in other study using adult rats [59].

The mitochondrial respiratory chain consists of a series of electron carriers that function as redox pairs involved in ATP generation, but its operation also increases ROS production [62, 63]. These reactive species may damage a variety of cell macromolecules, including those that constitute the electron transport system, therefore disrupting mitochondrial function in a vicious cycle, such that the oxidative damage induced by stress may be either the cause or the consequence of the mitochondrial dysfunction [64–66]. According to our findings, isolation stress increased the activity of complex IV in both ages groups (juvenile and adult), while the palatable diet reduced the activity of complex IV only in adulthood. Some studies [25, 67] have shown that stress exposure decreases the activity of respiratory complexes; in those studies, however, the type and intensity of stress used was different, as well as the age when it occurred. In the present study, stress exposure was applied during development, and the effects remained until adulthood. Glucocorticoids (GCs), which are released during the stress response, can directly or indirectly affect mitochondrial functions [16], and the cellular energy state was shown to be regulated by GCs via glucocorticoid receptors that regulate both nuclear and mitochondrial genes involved in respiratory enzyme biosynthesis [68]. Recently, a study showed that animals subjected to chronic stress increased the expression of cytochrome oxidase 1 (COX1) and cytochrome oxidase 3 (COX3) genes in prefrontal cortex [69], which are catalytic subunits of cytochrome *c* oxidase, the last enzyme in the respiratory electron transport chain of mitochondria [70, 71] which was also affected by stress exposure in the

present study. Therefore, it is possible that this effect on Complex IV activity is due to GCs-induced expression of these enzymes. Interestingly, there was no change in the mitochondrial mass or in the mitochondrial potential, indicating that stress was not able to change mitochondria quantity despite the complex IV increase. More studies are necessary to clarify the physiological impact and the causes of the complex IV activity increases without major alterations in mitochondrial quantity and general activity.

In conclusion, our findings showed that isolation stress in the prepubertal period can to lead long-lasting neurochemical changes in the prefrontal cortex of male rats and that a palatable diet was not able to reverse the stress-induced effects. This study also points to the importance of knowing the environmental factors which may influence stages of development, in order to better understand future neural consequences.

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CAPÍTULO 3

Manuscrito: Isolation stress exposure and consumption of palatable diet during the prepubertal period leads to cellular changes in the hippocampus

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ORIGINAL PAPER

Isolation Stress Exposure and Consumption of Palatable Diet During the Prepubertal Period Leads to Cellular Changes in the Hippocampus

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Abstract Social isolation is one of the most potent stressors in the prepubertal period and may influence disease susceptibility or resilience in adulthood. The glucocorticoid response and, consequently, the adaptive response to stress involve important changes in mitochondrial functions and apoptotic signaling. Previous studies have shown that consumption of a palatable diet reduces some stress effects. Therefore, the aim of the present study was to investigate whether isolation stress in early life can lead to cellular alterations in the hippocampus. For this, we evaluated oxidative stress parameters, DNA breakage index, mitochondrial mass and potential, respiratory chain enzyme activities, apoptosis, and necrosis in the hippocampus of juvenile male rats submitted or not to isolation stress during the pre-puberty period. We also verified whether consumption of a palatable diet during this period can modify stress effects. Results show that stress led to an oxidative imbalance, DNA breaks, increased the mitochondrial potential and early apoptosis, and decreased the number of live and necrotic cells. In addition, the palatable diet increased glutathione peroxidase activity, high mitochondrial potential and complex I–III activity in the hippocampus of juvenile rats. The administration of a palatable diet during the isolation period prevented the stress effects that caused the reduction in live cells and increased apoptosis. In conclusion, the stress experienced during the pre-pubertal period induced a hippocampal oxidative imbalance, DNA damage, mitochondrial dysfunction, and increased apoptosis, while consumption of a palatable diet attenuated some of these effects of exposure, such as the reduction in live cells and increased apoptosis, besides favoring an increase in antioxidant enzymes activities.

Keywords Hippocampus · Palatable diet · Isolation stress · Apoptosis · Mitochondria · Oxidative stress

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Introduction

The prepubertal period is a time of transition, sexual maturation, and enhanced brain architecture plasticity. In addition, this period is critical for the final maturation of circuits controlling energy homeostasis and stress responses [1]. Exposure to stressors in this phase of life may influence disease susceptibility or resilience in adulthood [2]. One of the most potent stressors, during this period, is social isolation [3], which can lead to behavioral, anatomical and neurochemical changes that may also be present in adulthood, when these animals are compared to their socially-reared litter mates (socials) [4, 5].

The exposure to stressful events leads to an increased release of glucocorticoids (GCs) due to activation of the hypothalamo-pituitary adrenal (HPA) axis. Additionally, glucocorticoid receptors (GR) mediate the negative feedback of GCs on the HPA axis following stress. These receptors are localized in distinct brain structures, including the hippocampus, prefrontal cortex and hypothalamus; however GRs are more abundant in the hippocampus,

which is the most stress-sensitive brain region [6, 7]. Although the stress response is essential and adaptive to changes in the environment, when this response is exaggerated or sustained, it may cause neuroendangerment, which is observed after prolonged stress exposure or in the presence of elevated GCs levels, and has been linked to an increased generation of reactive oxygen species (ROS) [8]. At low concentrations, ROS are involved in the regulation of a number of physiological processes, whereas their overproduction results in oxidative injury, which may mediate damage to cell structures such as lipid membranes, protein and DNA [9]. The imbalance between high cellular levels of ROS in relation to cellular antioxidant defenses, namely oxidative stress, may be involved in the pathogenesis of several brain diseases [10–14]. Previous studies from our laboratory have reported that social isolation in the prepubertal period leads to an imbalance in the antioxidant enzyme systems in the prefrontal cortex in juvenile rats [15], suggesting the vulnerability of the central nervous system to this type of stressor.

The adaptive response to stress involves important changes in mitochondrial functions, enabling organisms to adjust bioenergetics, thermogenesis, oxidative and/or apoptotic responses [16]. Apoptosis has also been proposed as a cellular mechanism that contributes to the structural changes observed in stress-related mood disorders in both human and animal models [17]. The apoptotic process is programmed and critically controlled by the balance between proapoptotic and antiapoptotic proteins in the outer mitochondrial membrane [18, 19], leading to a cascade of signals that ultimately results in cell death. In response to death stimuli, the mitochondria are impaired, resulting in the release of proapoptotic proteins, such as cytochrome *c* from the mitochondria into to the cytoplasm [20].

Increased stress has been associated with an increased motivation for foods rich in lipids and carbohydrates [21], and a model of reward-based eating has been suggested as a means to reduce the stress response [22]. Thus, it has been suggested that there may exist an interaction between the effects of stress and the consumption of easily-available palatable food [23]. On the other hand, some studies have presented evidence that the production of ROS and excessive intake of palatable foods can lead to breaks in cellular DNA [24–26]. Therefore, the aim of this study was to investigate the possibility that isolation stress in early life can lead to cellular alterations in the hippocampus. For this, we evaluated oxidative stress parameters, mitochondrial mass and membrane potential, respiratory chain enzyme activities, apoptosis and necrosis, and the DNA break index in the hippocampus of juvenile rats subjected, or not, to isolation. We also verified whether the consumption of a palatable diet could reduce stress effects during the prepubertal period.

Materials and Methods

Subjects

All animal procedures were approved by the Institutional Ethical Committee (CEUA-UFRGS 20040) and followed the recommendations of the Federation of the Brazilian Societies for Experimental Biology. All efforts were made to minimize animal suffering, as well as to reduce the number of animals.

Forty male Wistar rats, from 10 litters, were weaned on postnatal day 21. A total of four male pups were used from each litter, and these were divided into four groups, as described below, in such a way that one animal per litter was used in each group. Male pups were weighed and distributed into four groups, receiving a highly palatable diet or standard lab chow. Half of the animals were housed in groups of 5; the other half were stressed by isolation (one animal in a smaller home cage, 27 × 17 × 12 cm) [27], resulting in four experimental groups: controls receiving standard lab chow, controls receiving a palatable diet, isolated animals receiving lab chow and isolated animals receiving a palatable diet. These interventions occurred between postnatal days 21–28 and daily food consumption was measured. At postnatal day 28, the animals were killed by decapitation and biochemical evaluations were performed in the hippocampus. Half of the animals in each group were used for evaluation of oxidative stress parameters and respiratory chain enzymes activities, and the other half for determination of mitochondrial mass and membrane potential, as well as for measurement of the amount of live cells, cells in early apoptosis, late apoptosis and necrotic cells, and the DNA break index.

Palatable Diet

The palatable diet used on this study was enriched with simple carbohydrates, and was made with condensed milk, sucrose, vitamins and salts mix, powdered standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet was similar to that of a standard lab chow (including 22 % protein and 4–6 % fat), however most carbohydrates in the palatable diet were sucrose; in contrast, carbohydrates in the standard lab chow were provided mainly from starch (see Table 1).

Assessment of Oxidative Stress Parameters

The animals were killed by decapitation and their hippocampus was quickly dissected out and stored at –70° C until analysis. Structures were then homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer

Table 1 Composition of the diets used (in 100 g of each diet)

	Standard chow	Palatable diet
Carbohydrates	49 (starch)	41.4 (14.7 from starch, 26.7 from sucrose and lactose)
Fat	4	6.5
Protein	22	24.9

The remainder of these diets was constituted by vitamins, fibers, and minerals

(pH 7.4), containing 1 mM EDTA. The homogenate were centrifuged at 1,000×*g* for 10 min at 4° C and the supernatants were used.

Superoxide Dismutase Activity

Superoxide dismutase activity was determined using the RANSOD kit (Randox Labs., USA), which is based on a procedure described by Delmas-Beauvieux et al. [28]. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye, which can be assayed spectrophotometrically at 492 nm, at 37 °C. The inhibition of the production of the chromogen is proportional to the activity of SOD present in the sample; one unit of SOD causes 50 % inhibition of the rate of reduction of INT under the conditions of the assay.

Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined according to Wendel [29] with modifications. The reaction was carried out at 37 °C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg of protein.

Catalase Activity

Catalase activity assessment is based upon establishing the rate of H_2O_2 degradation spectrophotometrically at 240 nm at 25 °C [30]. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Evaluation of Free Radical Production by the Chemical Oxidation of Dichlorodihydrofluorescein (DCFH)

Samples were incubated with 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA 100 µM) at 37 °C for 30 min. DCFH-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen/nitrogen species. The formation of the fluorescent derivative dichlorofluorescein (DCF) was monitored by excitation and emission wavelengths of 488 and 525 nm, respectively, using a SpectraMax M5. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein [31].

Determination of Total Thiol Content

This assay measures protein and non-protein thiols; the latter is mainly represented by the reduced form of glutathione and is based in the reduction of 5,50-dithiobis 2-nitrobenzoic acid (DTNB) by thiol groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB) whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to the oxidative damage to proteins. Results are reported as nmol TNB/mg protein [32].

Comet Assay

A standard protocol for Comet assay preparation and analysis was followed [33]. The slides were prepared by mixing 20 µL of hippocampus dissociated (in cold PBS pH 7.4) with 80 µL low melting point agarose (0.75 %). The mixture (cells/agarose) was added to a fully frosted microscope slide coated with a layer of 500 µL of normal melting agarose (1 %). After solidification, the cover slip was gently removed and the slides were placed overnight in lysing solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, with freshly added 1 % Triton X-100 and 10 % dimethylsulfoxide [DMSO]). Subsequently, the slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH 12.6) for 5 min. The DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA under alkaline conditions (pH > 13). Subsequently, the slides were neutralized with 0.4 M Tris (pH 7.5) three times during 5 min. Finally, the DNA was stained with Syber Green. The stained nuclei were blindly analyzed by fluorescence microscopy with visual inspection (200X). Cells were scored from zero (no breaks observed) to 4 (maximal break index), according to the tail intensity (size and shape), resulting in a single DNA break score for each cell, and consequently, for each group. The DNA break index was calculated by multiplying the number of cells by

its respective index score and then summing it up. Therefore, a group index could range from zero (all cells with no tail, 100 cells X 0) to 400 (all cells with maximally long tails, 100 cells X 4) [34].

Mitochondrial Mass and Membrane Potential Measurement

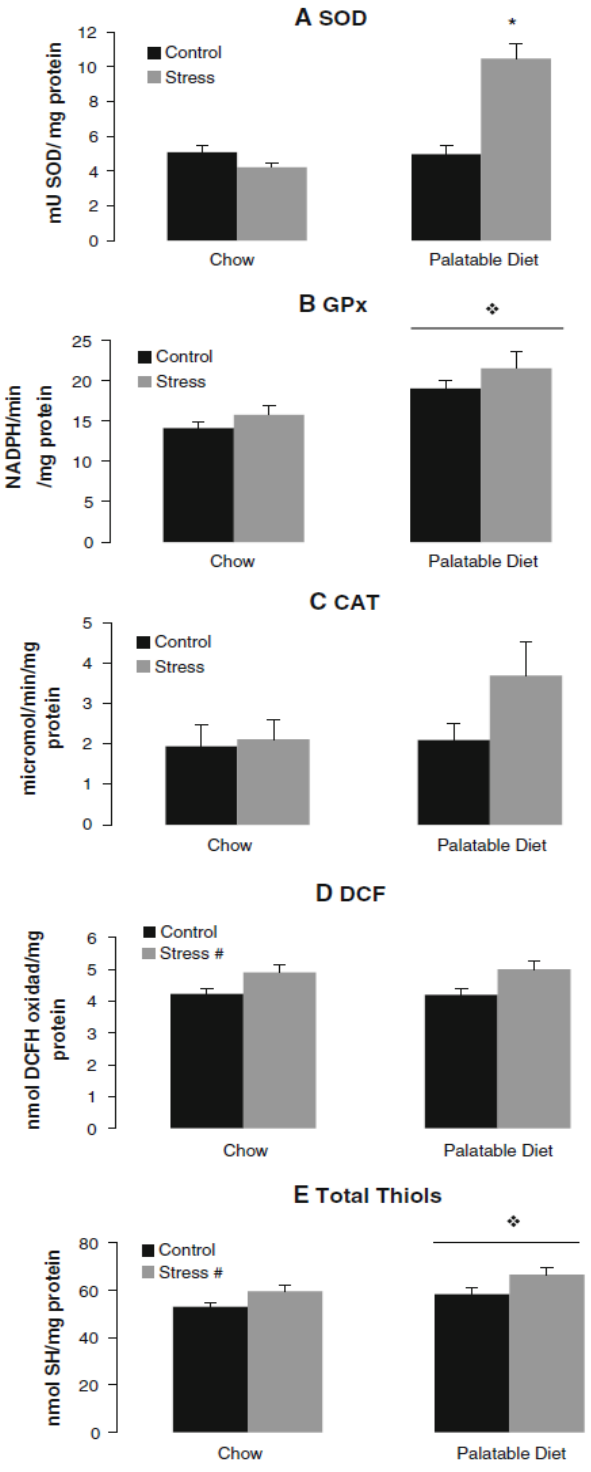
Mitotracker was used for mitochondrial function analysis in cell suspensions of hippocampus obtained by mechanical dissociation with PBS containing collagenase to favor digestion to a density of about 200,000 cells/mL. The dissociated contents were then filtered through 40 µm nylon cell strainers. To assess mitochondrial potential ($\Delta\psi$) and mass, MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG) dyes were employed [35, 36]. MTG is a green-fluorescent fluorophore that accumulates in mitochondria regardless of mitochondrial membrane potential and has been used to measure mitochondrial mass [37]. MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock concentration. Samples were prepared as described by Weis et al. [38]. Immediately after staining, cell suspensions were analyzed by flow cytometry. MitoTracker dyes were excited using a 488 air-cooled argon laser. Negative controls (samples without stain) were included for setting up the machine voltages. Controls stained with a single dye were also employed to allow the setting of compensation. The emission of fluorochromes was recorded through detectors and specific band-pass fluorescence filters: red (FL3; 670 nm long pass) and green (FL1; 530 nm/30). All flow cytometric acquisitions and analyses were performed using CELLQuest Pro data acquisition (BD Biosciences) and FlowJo analysis software, respectively. The histograms were divided into two halves (named cells with low and high mass or potential), based on the peak of the controls for MTG and MTR, and this evaluation was extended to all data in each parameter. Using this method, analyses of cells resulted in two populations, with different mitochondrial mass and $\Delta\psi$. The lower accumulation of MTG or MTR, and thus lower fluorescence values, are indicative of decreased mitochondrial mass or $\Delta\psi$ [35, 36].

Respiratory Chain Activity Determination

Brain structures were freshly homogenized (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base), pH 7.4, for determination of respiratory chain complex activities. The homogenates were centrifuged at 1,000 g for 10 min at 4 °C and the supernatants were immediately maintained at −70 °C until analyses. The activities of the electron transport chain (ETC) complexes I–III, II and IV were determined according to standard methods previously described in the literature [38–41]. The activity of complex I–III (complex I + CoQ + III) was assessed by measuring the increase in absorbance due to cytochrome *c* reduction at 550 nm, according to the method described by Schapira et al. [41]. Complex I–III activity was calculated as the rotenone sensitive NADH: cytochrome *c* reductase activity. The activity of complex II (succinate: DCIP oxyredutase) was determined according to Fischer et al. [39], by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm, in a medium containing sodium succinate, sodium azide, and rotenone and DCIP. Cytochrome *c* oxidase (COX, complex IV) activity was determined, according to Rustin et al. [40], following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm. The activities of the respiratory chain complexes were calculated and expressed as nmol per min per mg of protein.

Determination of Live cells, Early Apoptosis, Late Apoptosis and Necrosis

These parameters were analyzed using cell suspensions of hippocampus. Hippocampus was mechanically dissociated with PBS containing collagenase to obtain a density of 200,000 cells/mL, and filtered through a 40-µm nylon cell strainer. The live/dead cell assay was performed according to the manufacturer's instructions (Apoptotic, Necrotic & Health Cells Qualification kit-Uniscience). To assess apoptosis (early apoptosis), dead cells by apoptosis (late apoptosis), necrosis and live cells, specific fluorescent probes (annexin V for apoptotic cells and ethidium homodimer to detect dead cells by necrosis) were employed. Briefly, 10^6 cells were incubated at room temperature with annexin V, ethidium homodimer (EH) and binding buffer for 15 min. The levels of annexin incorporation and levels of EH positive cells were determined by flow cytometry (FACSCalibur, Becton–Dickinson, Franklin Lakes, NJ, USA). FITC and EH dyes were excited at 488 nm using an air-cooled argon laser. Negative controls (samples without label) were included for setting up the machine voltages. Controls stained with a single dye (FITC and EH) were used to set compensation. The emission of fluorochromes was recorded through detectors using specific band-pass fluorescence filters: green (FL1; 530 nm/30) and red (FL3; 670 nm long pass) and collected using logarithmic amplification. Flow cytometry data were analyzed and plotted by density as a dot plot, which shows the relative FL1 fluorescence on the y-axis and the relative FL3 fluorescence on the x-axis. The quadrants to determinate the negative and positive area were placed based on control samples with 80–90 % live cells and this setting was applied to all samples. The number of cells in each quadrant was computed and the proportion of negatively-stained



◀ **Fig. 1** Effects of isolation stress and consumption of palatable diet on antioxidant enzyme activities, free radical (DCFH test) production and on total thiol levels in the hippocampus of juvenile rats. Data are expressed as mean + SEM N = 4–5/group. **a** SOD (expressed as U/mg protein), **b** GPx (expressed as nmol NADPH consumed/min/mg protein), **c** CAT (expressed as micromoles of H_2O_2 consumed/min/mg protein), **d** DCFH (expressed as nmol of DCF formed/mg protein) and **e** total thiols (expressed as nmol TNB/mg protein). *Interaction between stress and palatable diet (two-way ANOVA, $P < 0.001$). #Stress increased free radical production (two-way ANOVA, $P < 0.01$) and total thiol levels (two-way ANOVA, $P < 0.02$). ❖Palatable diet increased GPx activity (two-way ANOVA, $P < 0.001$) and total thiol levels (two-way ANOVA, $P < 0.05$)

cells or cells stained with annexin, EH or both were expressed as live, apoptotic, necrotic or cells in late apoptosis, respectively [42–45].

Protein Assay

The protein concentration was determined in the samples using the method described by Lowry et al. [46] with bovine serum albumin as the standard.

Statistical Analysis

Data were expressed as mean ± SE of the mean, and were analyzed using two-way ANOVA, with isolation stress and palatable diet as factors. All analyses were performed using SPSS software and a $P < 0.05$ was considered significant.

Results

Antioxidant Enzyme Activities, Free Radical Production and Total Thiol Levels

These parameters were analyzed to verify whether there was an oxidative imbalance in the hippocampus of rats exposed to isolation stress and palatable diet in the prepuberty period. When evaluating SOD activity (Fig. 1a) at 28 days (juveniles), an interaction between stress and palatable diet was observed [F (1,16) = 35.64, $P < 0.001$], shown by the potentiation of SOD activity in the presence of both factors. In contrast, the palatable diet induced an increase in GPx activity [F(1,16) = 16.63, $P < 0.001$] (Fig. 1b), while CAT activity (Fig. 1c) was not altered by isolation stress or by access to a palatable diet ($P > 0.05$). Additionally, isolation stress increased free radical production [F (1,16) = 10.00, $P < 0.01$], as evaluated using the DCFH test (Fig. 1d). Both isolation stress [F (1,16) = 8.18, $P < 0.02$] and palatable diet [F (1,16) = 5.66, $P < 0.05$] increased total thiol levels (Fig. 1e).

Table 2 Effects of isolation stress and consumption of palatable diet during the prepubertal period on *SOD/GPx* and *SOD/CAT* ration in the hippocampus of juvenile rats. Data are expressed as mean ± SEM

Group	*SOD/GPx* ratio	*SOD/CAT* ratio
Control + chow	0.36 ± 0.023	4.46 ± 1.86
Control + palatable diet	0.26 ± 0.026	2.75 ± 0.59
Stressed + chow	0.27 ± 0.036	2.39 ± 0.49
Stressed + palatable diet	0.49 ± 0.038*	4.86 ± 2.45

* Interaction stress × diet: increased this ratio (two-way ANOVA, $P < 0.001$). N = 5/group

The SOD/GPx ratio (Table 2) showed an interaction between isolation stress and palatable diet [F (1,16) = 24.46, $P < 0.001$], while there was no difference in the SOD/CAT ratio.

DNA Break Index

As shown in Fig. 2, isolation stress exposure increased the DNA break index [F (1,42) = 12.37, $P < 0.001$] in the hippocampus of juvenile rats; the palatable diet had no effect on this parameter.

Mitochondrial Mass and Membrane Potential

Figures 3 and 4 show hippocampal cells labeled with MTG and MTR, respectively. Isolation stress decreased the number of cells presenting low mitochondrial mass [F (1,15) = 11.09, $P < 0.01$] (Fig. 3a) and low mitochondrial $\Delta\psi$ [F (1,15) = 9.67, $P < 0.01$] (Fig. 4a). No difference in high mitochondrial mass was observed in the cells, $P > 0.05$ (Fig. 3b).

Both isolation stress [F (1,15) = 7.74, $P < 0.05$] and palatable diet [F (1,15) = 5.18, $P < 0.05$] (Fig. 3b) increased the number of cells presenting high $\Delta\psi$.

Respiratory Chain Enzyme Activities

Mitochondrial energy metabolism in the hippocampus was evaluated using enzymatic analysis of electron transport chain (ETC.) activities (Table 3). The consumption of a palatable diet during the prepubertal period increased complex I + III activity [F (1,14) = 5.02, $P < 0.05$]. In contrast, the enzyme activities of Complex II and Complex IV were not significantly altered ($P > 0.05$).

Flow Cytometry Analysis After Annexin V and Ethidium Homodimer Staining

As shown in Fig. 5, stress decreased the number of live cells [F (1,16) = 4.48, $P = 0.05$] and there was an interaction between stress and palatable diet [F (1,16) = 7.22,

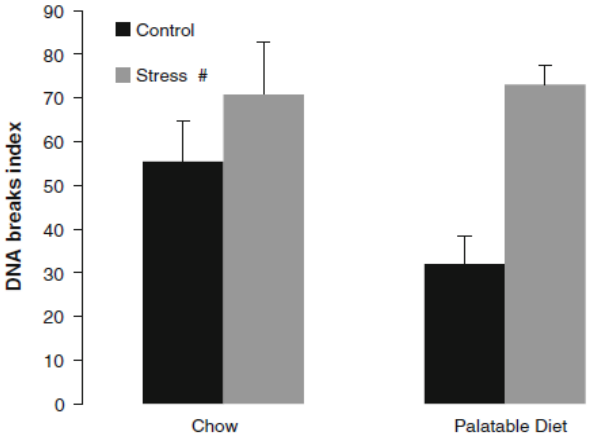


Fig. 2 Effect of isolation stress and consumption of palatable diet during the prepubertal period on comet assay (expressed as breakage index), in the hippocampus of juvenile rats. Data are expressed as mean + SEM N = 5/group. #Stress significantly increased DNA breakage index (two-way ANOVA, $P < 0.01$)

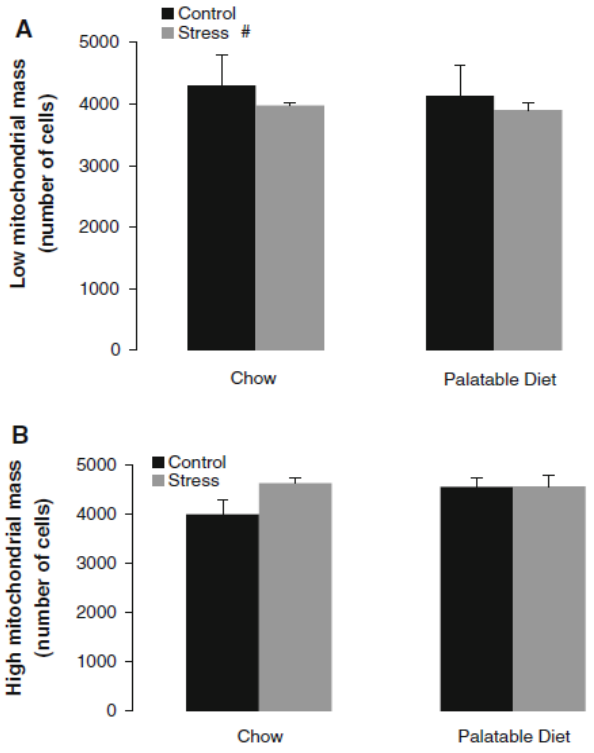


Fig. 3 Effects of isolation stress and consumption of palatable diet in cells labeled with Mitotracker Green (mitochondrial mass) in the hippocampus of juvenile rats. Data are expressed as mean ± SEM N = 4–5/group. **a** Low mass, **b** High mass. #Stress decreased low mitochondrial mass (two-way ANOVA, $P < 0.01$)

Table 3 Effects of isolation stress and consumption of palatable diet during the prepubertal period on respiratory chain enzyme activities (nmol/min/mg protein) in the hippocampus of juvenile rats

Group	Complex I–III	Complex II	Complex IV
Control + chow	5.05 ± 0.34	6.48 ± 0.49	47.98 ± 6.25
Control + palatable diet	6.25 ± 0.73❖	6.50 ± 0.88	50.9 ± 7.28
Stressed + chow	5.04 ± 0.16	7.39 ± 1.22	53.27 ± 2.03
Stressed + palatable diet	6.05 ± 0.55❖	7.53 ± 0.62	46.92 ± 4.06

Data are expressed as mean ± SEM of cytochrome c reduction (Complex I + III), 2,6-DCIP reduction (Complex II) and oxidation of previously reduced cytochrome c (Complex IV)

❖ Palatable diet increased complex I–III activity (two-way ANOVA, $P < 0.05$). N = 4–5/group

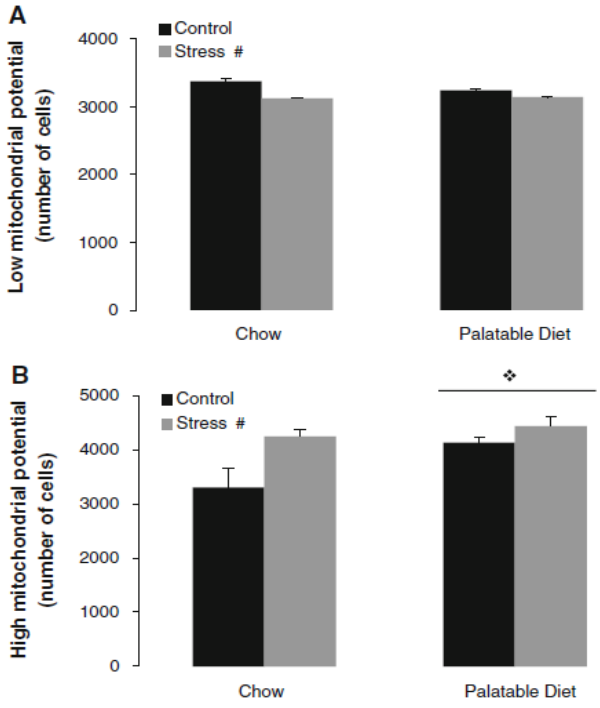


Fig. 4 Effects of isolation stress and consumption of palatable diet in cells labeled with Mitotracker Red (mitochondrial potential) in the hippocampus of juvenile rats. Data are expressed as mean ± SEM N = 4–5/group. **a** Low potential, **b** High potential, #Stress decreased low mitochondrial potential (two-way ANOVA, $P < 0.01$) and increased high mitochondrial potential (two-way ANOVA, $P < 0.05$). ❖Palatable diet increased high mitochondrial potential (two-way ANOVA, $P < 0.05$)

$P < 0.02$] (Fig. 4a), since the stressed group that received the palatable diet did not demonstrate the same decrease in the number of live cells. Additionally, exposure to isolation stress induced an increase in the number of positive cells labeled with annexin-V (early apoptosis; Fig. 5b) [F (1,16) = 18.57, $P < 0.001$] and an interaction between stress and palatable diet was also observed for this parameter [F (1,16) = 8.06, $P < 0.02$]. No significant difference was observed in late apoptosis (double labeling; $P > 0.05$) (Fig. 5c).

With regard to necrosis, stress decreased the number of cells stained with EH [F (1,16) = 9.99, $P < 0.01$] and an interaction between stress and diet was also observed [F (1,16) = 5.08, $P < 0.05$] (Fig. 5d), further decreasing this number (Fig. 5).

Discussion

The data of the present study demonstrate that isolation stress, during the prepubertal period, induces neurochemical changes by increasing SOD activity, free radical production, DNA breaks, total thiol content, early apoptosis, high mitochondrial membrane potential and reducing the number of live and necrotic cells, as well as cells with low mitochondrial mass, in the hippocampus. These results suggest stress-induced neuroendangerment. Additionally, free access to a palatable diet was found to prevent some of these stress-induced effects in the hippocampus of juvenile rats, including the alteration in the numbers of live and apoptotic cells. These data are in accordance with previous studies suggesting that the consumption of palatable food, rich in fat and carbohydrates, may decrease the stress response in chronically-stressed rats [23]. On the other hand, the increased SOD/GPx ratio observed in stressed animals receiving the palatable diet represents an imbalance in antioxidant defenses and suggests that this association may sometimes be inadequate.

The brain is especially vulnerable to free radical production and to oxidative damage due to its high oxygen consumption, abundant lipid content and a relative paucity of antioxidant enzymes [47–49]. Several studies have reported that stress results in the imbalance of the antioxidant status, which ultimately leads to increased oxidative stress [50–52]. Based on these considerations, we evaluated oxidative stress parameters, such as antioxidant enzymes activities (SOD, GPx and CAT), free radical production (DCFH oxidation test) and total thiol content. Isolation stress increased the production of free radicals, the total thiol content and decreased SOD activity. However, despite the decreased *SOD/GPx* ratio, no differences in GPx and CAT activities were observed following isolation stress. Since SOD dismutates the superoxide ion, generating hydrogen peroxide (H_2O_2), and GPx and CAT degrade H_2O_2 into water (H_2O), any changes in this system can lead to an imbalance in antioxidant defenses. In fact, superoxide and H_2O_2 production has been reported to be enhanced

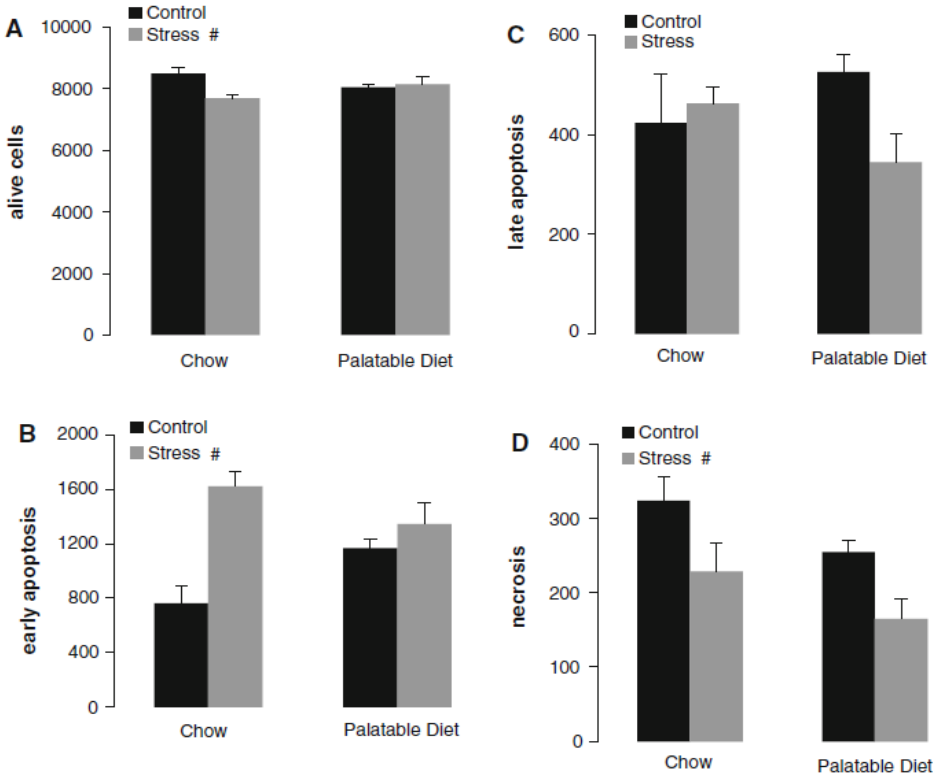


Fig. 5 Effects of isolation stress and consumption of palatable diet on live cells, apoptosis, late apoptosis and necrosis in the hippocampus of juvenile animals. Data are expressed as mean ± SEM N = 4–5/group. **a** Live cells (Annexin V^-/EH^-), **b** Early Apoptosis (Annexin V^+/EH^-) **c** Late apoptosis (Annexin V^+/EH^+) and **d** Necrosis (Annexin V^-/EH^+). #Effect of isolation stress (two-way ANOVA). There was also an interaction between stress and palatable diet for the number of live cells, cells in early apoptosis and necrotic cells

after stress [53]. Our data suggest an oxidative imbalance, as there was an increase in the production of free radicals. In addition, the isolation stress increased total thiol content, which may be associated with increased levels of thiols from proteins or a reduction in glutathione (a substrate for GPx); however, this increase was not accompanied by increased GPx activity.

Access to a palatable diet increased SOD activity and there was an interaction between isolation stress and palatable diet, with a potentiation of this enzyme activity. However, this diet also increased total thiol content and GPx activity, favoring a more general increase in the activity of antioxidant enzymes, in contrast to observations in the stressed group. Additionally, when both factors (stress and diet) were applied simultaneously, there was an increase in the SOD/GPx ratio.

In addition to causing an imbalance in antioxidant defenses, isolation stress was able to increase the break index. DNA breaks were measured by the comet assay under alkaline conditions; this assay can detect single and double-stranded breaks, incomplete repair sites, alkali labile sites and possibly both DNA–protein and DNA–DNA cross-links in eukaryotic cells [54]. Previous investigations have reported that generation of ROS and induction of DNA damage in a cell may trigger mitochondria-mediated apoptosis [55, 56]. In addition, apoptosis can induce DNA fragmentation [57]. It is possible that the increase in DNA breakage and the increase in ROS production may be related to an induction of apoptosis. It is important to point out, however, that DNA breaks, measured by this method, may be correctly repaired without resulting in permanent genetic alterations [58].

There were no palatable diet-induced changes in DNA breakages in the hippocampus of juvenile rats. In contrast to these results, some studies have presented evidence that excessive intake of palatable foods can lead to breaks in cellular DNA [26, 59]. This discrepancy may be explained by the age of the animals when exposed to the diet, its duration, and the type of palatable diet. For example, during the prepubertal period, high fat diet induced DNA damage in rats [26]; chronic consumption of chocolate in adult rats was also able to induce DNA breaks [59]. Therefore, it is possible that animals subjected to high-fat diets may be more prone to present DNA breakages in their cells than animals subjected to a diet enriched in simple carbohydrates, as used in the present study. Alternatively, it is also possible that a longer period of exposure to this diet (enriched in simple carbohydrates) could lead to a different outcome for this parameter.

The increased production of free radicals, with an altered SOD/GPx ratio, as observed in this study, prompted us to study the mitochondrial membrane potential, since it plays a crucial role in the generation of superoxide radicals. We then studied some aspects of mitochondrial function, since the mitochondria generates a large quantity of reactive oxygen species (ROS) [60]. We evaluated mitochondrial mass and membrane potential, as well as respiratory complex activities. Isolation stress led to a decrease in cells with low mitochondrial mass, and it also increased the number of cells with higher mitochondrial $\Delta\psi$, but without any change in respiratory complex activities. The increase in the high mitochondrial $\Delta\psi$ with a decrease in cells with low mitochondrial mass could suggest a possible mitochondrial biogenesis; however no alterations in respiratory complex activities were observed. Another possible explanation for the increased number of cells with higher mitochondrial $\Delta\psi$ may be associated with the oxidative imbalance observed in this study. Interestingly, the rate of ROS emission is strongly controlled by the mitochondrial membrane potential; increased membrane potential is associated with increased ROS emission rate and decreased active phosphorylation [61]. Therefore, the increased mitochondrial potential could help to explain the oxidative imbalance observed in this study in stressed animals. On the other hand, consumption of palatable diet induced an increase in the number of cells with higher mitochondrial $\Delta\psi$ and also in the complex I–III activities. It is possible that this increase is an adaptive response induced by the fact that more glucose is being oxidized in the tricarboxylic acid cycle, which in effect pushes more electron donors into the electron transport chain. In this case, although we observed an increased mitochondrial potential, both GPx and SOD activities were increased and no increased free radical production was observed, in contrast to the results observed for isolated animals.

Alterations in mitochondrial physiology and oxidative stress-induced cellular damage are considered to constitute a central event in apoptosis [62–64]. In the present study, we measured the number of cells stained with annexin V, which identifies apoptotic cells, and we also analyzed the number of cells stained with ethidium homodimer, used to detect dead cells by necrosis. Isolation stress reduced the number of live cells (without staining) and necrotic cells, and increased the number of the cells stained with annexin V, indicating that this type of stress in the prepubertal period induces early apoptosis. The consumption of palatable food prevented both of these stress-induced effects, the reduction in the number of live cells, as well as the increase in early apoptosis, in agreement with the hypothesis that palatable food consumption may reduce stress effects [23]. During apoptotic cell death, evidence indicates an increase in mitochondrial function in a number of cell types [65–67], although other studies have shown a link between a decrease in the mitochondrial membrane potential and apoptosis [68–70]; furthermore, it is possible that this parameter may depend on the stage of apoptotic cell death. During the prepubertal period, intense modifications occur in the nervous systems [1], and the increased number of cells during early apoptosis and reduced number of necrotic cells may suggest some type of reorganization of circuits induced by isolation. Other studies are necessary to better understand these effects of isolation during the prepubertal period.

In conclusion, the present study shows that isolation stress during the prepubertal period induces early apoptosis, and increases mitochondrial potential, oxidative imbalance and DNA breakage, suggesting cellular damage. Consumption of a palatable diet enhanced antioxidant enzymes activities and prevented stress effects on early apoptosis and on the number of live cells, indicating that the access to a palatable diet during exposure to isolation may function as a compensatory mechanism or may facilitate the adaptation to stress during the prepubertal period. However, other effects of stress exposure were not prevented by the use of a palatable diet. This study also indicates the importance of environmental factors, which may influence plasticity or neuronal susceptibility during different stages of development. More studies are needed to clarify whether these effects are long-lasting and how they can influence adult/senile brain function.

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CAPÍTULO 4

Manuscrito: Long-lasting effects of palatable diet during prepubertal stress isolation on behavior and hippocampal oxidative balance in adult rats.

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Manuscript Draft

Manuscript Number:

Title: LONG-LASTING EFFECTS OF PALATABLE DIET DURING PREPUBERTAL STRESS ISOLATION ON BEHAVIOR AND HIPPOCAMPAL OXIDATIVE BALANCE IN ADULT RATS

Article Type: Research Article

Keywords: hippocampus; palatable diet; isolation stress; oxidative stress; glucocorticoid receptor; memory.

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Abstract: During prepubertal period, environmental changes, including diet and stress exposure, are increasingly recognized as determinants of either adaptive or maladaptive brain functioning. The aim was to investigate whether isolation stress and intake of palatable diet in early life can lead to long-term alterations in behavior and hippocampal neurochemical parameters in adult male rats. We evaluated spatial memory, motor activity, and neurochemical parameters (oxidative stress, DNA fragmentation index, mitochondrial mass and potential, respiratory chain enzymes activities, apoptosis, necrosis, glucocorticoid receptors, and BDNF levels) in the hippocampus of adult male rats exposed or not to isolation stress and a palatable diet during pre-puberty. The isolation and palatable diet did not affect spatial memory, but reduced swimming speed. In addition, the isolation induced long-lasting changes in the oxidative balance of hipppocampus characterized by increased reactive species generation and the SOD:CAT and SOD:GPx ratios, which are possibly related to the increased Complex IV activity and increased necrosis. Interactions (isolation and palatable diet) were observed in some of the parameters evaluated, which returned to control levels: respiratory enzymes activities, DNA fragmentation index and the number of necrotic and in late apoptosis cells. These effects of palatable diet could be related to the observed increase in glucocorticoid receptors in the hippocampus. However, the palatable diet also had some per se effects, such as increased SOD:GPx ratio and incresed DNA fragmentation and early apoptosis, as well as reduced number of live cells. Moreover, since the memory was not affected and mitochondrial dysfunctions are known to be involved with mood disorders, the behavioral changes observed seem to be related to motivational aspects rather than cognitive features, and could be attributed to changes in the neurochemical parameters evaluated. Therefore, stress and palatable diet during the prepubertal period may increase vulnerability of hippocampus during adulthood.

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LONG-LASTING EFFECTS OF PALATABLE DIET DURING PREPUBERTAL STRESS ISOLATION ON BEHAVIOR AND HIPPOCAMPAL OXIDATIVE BALANCE IN ADULT RATS

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ABSTRACT

During prepubertal period, environmental changes, including diet and stress exposure, are increasingly recognized as determinants of either adaptive or maladaptive brain functioning. The aim was to investigate whether isolation stress and intake of palatable diet in early life can lead to long-term alterations in behavior and hippocampal neurochemical parameters in adult male rats. We evaluated spatial memory, motor activity, and neurochemical parameters (oxidative stress, DNA fragmentation index, mitochondrial mass and potential, respiratory chain enzymes activities, apoptosis, necrosis, glucocorticoid receptors, and BDNF levels) in the hippocampus of adult male rats exposed or not to isolation stress and a palatable diet during pre-puberty. The isolation and palatable diet did not affect spatial memory, but reduced swimming speed. In addition, the isolation induced long-lasting changes in the oxidative balance of hipppocampus characterized by increased reactive species generation and the SOD:CAT and SOD:GPx ratios, which are possibly related to the increased Complex IV activity and increased necrosis. Interactions (isolation and palatable diet) were observed in some of the parameters evaluated, which returned to control levels: respiratory enzymes activities, DNA fragmentation index and the number of necrotic and in late apoptosis cells. These effects of palatable diet could be related to the observed increase in glucocorticoid receptors in the hippocampus. However, the palatable diet also had some per se effects, such as increased SOD:GPx ratio and incresed DNA fragmentation and early apoptosis, as well as reduced number of live cells. Moreover, since the memory was not affected and mitochondrial dysfunctions are known to be involved with mood disorders, the behavioral changes observed seem to be related to motivational aspects rather than cognitive features, and could be attributed to changes in the neurochemical

parameters evaluated. Therefore, stress and palatable diet during the prepubertal period may increase vulnerability of hippocampus during adulthood.

Key-words: hippocampus; palatable diet; isolation stress; oxidative stress; glucocorticoid receptor; memory.

1. Introduction

The prepubertal period is characterized by continuous development and brain plasticity (Pervanidou and Chrousos, 2012). In the natural environment, rodents live in groups and exhibit high levels of social behavior, both with younger and older animals (Panksepp et al., 2007). Social interactions are rewarding (Panksepp and Lahvis, 2007), while social isolation is an aversive event and increases the activity of the hypothalamo-pituitary adrenal (HPA) axis (Douglas et al., 2004; McCormick and Mathews, 2007). Social isolation is one of the most potent stressors during development (Arakawa, 2005), and at long-term it may induce a variety of behavioral abnormalities, including increased aggressiveness (Koike et al., 2009; Pinna et al., 2009; Pinna et al., 2003), anxiety-related behaviors (Pinna et al., 2006; Wei et al., 2007), cognitive deficits (Pibiri et al. 2008), hypoalgesia and hyperlocomotion (McEwen, 2007).

The exposure to stressful events leads to increased release of glucocorticoids (GCs) by acting on the HPA axis. Glucocorticoid receptors (GR) that also mediate the negative feedback of GCs on the HPA axis following stress, are located in distinct brain structures, mainly in the hippocampus, which is the most stress-sensitive region in the brain (McEwen, 2008; Sapolsky, 2003). Although the stress response is essential and adaptive to environmental changes, it may cause neuroendangerment when it is exaggerated or sustained. This neuronal impairment has been linked to the increased generation of reactive oxygen species (ROS), mitochondrial dysfunction (McIntosh and Sapolsky,1996), an altered production and/or release of neurotrophic factors (Pisu et al., 2011). All these changes may modify synaptic responses (Vereker et al., 2001), and hippocampal-dependent behavior, such as spatial learning and memory (Benzi et al., 1990; Fukui et al., 2002; Jhoo et al., 2004). In addition, changes in mitochondrial

physiology and oxidative stress-induced cellular damage constitute a central event in apoptosis (Franklin, 2011; Valko et al., 2006).

Increased stress has been associated with an increased motivation for foods rich in lipids and carbohydrates (Epel et al., 2001), and a model of reward-based eating has been suggested to reduce the stress response (Adam and Epel, 2007). Thus, there may be an interaction between the effects of stress and the intake of easily-available palatable food (Pecoraro et al., 2004). On the other hand, some studies have shown that the production of ROS and excessive intake of palatable foods can lead to fragmentation in the cellular DNA (Higashimoto et al., 2009; Muqbil et al., 2006; Olivo-Marston et al., 2008); and that The intake of such diets can be associated to cognitive decline and enhanced vulnerability to brain injury (Ansari et al., 2008; Baran et al., 2005; Srivareerat et al., 2009).

Previous studies from our laboratory have reported that social isolation in the prepubertal period leads to an imbalance in the antioxidant enzyme systems, mitochondrial dysfunction and an increase in cell death by apoptosis in the hippocampus of juvenile rats (Krolow et al., 2013), suggesting that the central nervous system is vulnerable to this type of stressor. The effects of social isolation early in life are partly prevented by palatable diet intake (Krolow et al., 2013). However, whether these stress-induced effects would remain during adulthood it is not known.

The present study aims to investigate if isolation stress in the prepubertal period can lead to long-term changes in behavior and hippocampal neurochemistry in adult male rats; and verified whether the access to a palatable diet during the same period would modify the effects of stress. To address these issues, we evaluated spatial memory, oxidative stress parameters and DNA fragmentation index, mitochondrial mass and potential, respiratory chain enzymes activities, apoptosis, necrosis,

immunocontent of GR and brain-derived neurotrophic factor (BDNF) levels in the hippocampus of adult male rats exposed to isolation stress during the prepubertal period taking or not the palatable diet.

2. Experimental procedures

2.1. Subjects

All animal proceedings were performed in strict accordance to the recommendations of the Brazilian Society for Neurosciences (SBNeC) and Brazilian Law on the use of animals (Federal Law 11.794/2008), and were approved by the Institutional Ethical Committee (CEUA-UFRGS 20040). All efforts were made to minimize animal suffering, as well as to reduce the number of animals used.

Forty male Wistar rats, from 10 litters, were weaned on postnatal day 21. A total of four male pups were used from each litter, and these were divided into four groups, as described below, in such a way that one animal per litter was used in each group. Male pups were weighed and distributed into four groups, receiving a high palatable diet or standard lab chow. Half of the animals were housed in groups of 5; the other half were stressed by isolation (one animal in a smaller home cage, 27x17x12 cm), resulting in the four experimental groups: controls receiving standard lab chow, controls receiving palatable diet, isolated animals receiving lab chow and isolated animals receiving palatable diet. These procedures were performed between postnatal days 21-28. After postnatal day 28, the animals received standard chow and were reared in groups of 5 per cage until adulthood. From postnatal day 60, behavioral analyzes were performed. Twenty-four hours after behavioral analysis, each animal was killed by decapitation and biochemical evaluations were performed in the hippocampus.

2.2 Palatable Diet

The high palatable diet used in this study was enriched in simple carbohydrates, and it was made with condensed milk, sucrose, vitamins and salts mix, powder standard lab chow, purified soy protein, soy oil, water, methionine and lysine. The nutritional content of this diet was similar to that of a standard lab chow (including 22% protein and 4-6% fat), however 41.4g% of carbohydrates contained in the palatable diet were represented by 26.7g% of sucrose and lactose and 14.7g% of starch; in contrast, the standard lab chow had 49g% carbohydrates, mainly from starch.

2.3 Spatial Memory Evaluation

The Morris water maze (Morris et al., 1982) is a behavioral test in which animals are required to find a submerged (1 cm) platform located at the center of a quadrant of the tank (a black circular pool with 200 cm in diameter and 100 cm high), using only distal, spatial cues available within the testing room. Rats are proficient, but reluctant swimmers and readily use the platform to escape the water. When animals were 2 months of age, they were submitted to daily sessions of four trials per day during 8 days to find the submerged platform. On each trial, the rat was placed in the water, facing the edge of the tank, at one of the four standard start locations (N, S, W and E). The order of the start locations varied in each sequence so that, for each block of four trials, any given sequence was not repeated on consecutive days. The rat was then allowed 60 s to search for the platform. Latency to find the platform (escape latency) was measured in each trial. Once the rat located the platform, it was allowed to remain on it for 15 s. If the rat did not find the platform within this time, it was guided to it and allowed to remain on it for 15 s. After each trial, the rats were removed, dried with a towel and put back in their home cages. The interval between trials was around 15 min

(Pettenuzzo et al., 2002). During the training sessions, the latency to find the platform was assessed in each trial.

On the 9th day, the animals were subjected to a probe trial that consisted of a single trial, with the platform removed. The latency to find the position where the platform originally was, the number of crossings on the original place of the platform, swimming speed and time spent in the target quadrant (where the platform used to be), as well as in the opposite quadrant, were measured.

2.4 Exposure to the open field

A 50-cm high, 40 x 60 cm open field made of wood with a frontal glass wall was used (Mello e Souza et al., 2000; Silveira et al., 2005). The floor was subdivided with white lines into 12 equal 13.3 x 15.0 cm rectangles, and the animals were gently placed facing the left corner and allowed to explore the arena for 5 min. The performance was observed, and line crossings were counted. Twenty-four hours later, the animals were subjected to the same task. The number of crossings of both days was used as a measure of motor activity.

2.5 Assessment of oxidative stress parameters

The animals were killed by decapitation. The hippocampus was quickly dissected out and stored at –70° C until analysis. Then, structures were homogenized in 10 vol (w:v) ice-cold 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA. The homogenate were centrifuged at 1000 x g for 10 min at 4° C and the supernatant were used.

2.5.1 Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined using the RANSOD kit (Randox Labs., USA) based on the procedure described by Delmas-Beauvieux et al. (1995). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye that is assayed by spectrophotometric analysis at 492 nm at 37°C. The inhibition in the production of the chromogen is proportional to the activity of SOD present in the sample; one unit of SOD causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

2.5.2 Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was determined according to Wendel (1981) with modifications. The reaction was carried out at 37°C in a solution containing 20 mM potassium phosphate buffer (pH 7.7), 1.1 mM EDTA, 0.44 mM sodium azide, 0.5 mM NADPH, 2 mM glutathione and 0.4 U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. GPx activity was expressed as nmol NADPH oxidized per minute per mg of protein.

2.5.3 Catalase Activity

Catalase (CAT) activity assessment is based upon establishing the rate of H_2O_2 degradation by spectrophotometric analysis at 240 nm at 25°C (Aebi, 1984). CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

2.5.4 Evaluation of free radicals production by the chemical oxidation of dichlorodihydrofluorescein (DCFH)

The samples were incubated with 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA 100 µM) at 37°C for 30 minutes. DCFH-DA was cleaved by cellular esterases and the DCFH formed was eventually oxidized by reactive oxygen/nitrogen species. The formation of the fluorescent derivative dichlorofluorescein (DCF) was monitored by excitation and emission wavelength of 488 and 525nm, respectively, using a SpectraMax M5. The amount of reactive oxygen/nitrogen species was quantified using a DCF standard curve and results were expressed as nmoles of DCF formed per mg of protein (Sriram et al. 1997).

2.5.5 Determination of Total Thiol Content

This assay measures protein and non-protein thiols; the latter is mainly represented by the reduced form of glutathione and the method is based in the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by thiol groups, which becomes oxidized (disulfide), yielding a yellow compound (TNB), whose absorption is measured by spectrophotometric analysis at 412 nm. The sulfhydryl content is correlated to reduced thiol groups (including reduced glutathione) and inversely correlated to the oxidative damage to proteins. Results are reported as nmol TNB/mg protein (Asksenov and MarKesbery, 2001).

2.6 Comet assay

A standard protocol for Comet assay preparation and analysis was followed (Tice et al., 2000). The slides were prepared by mixing 20 µL of hippocampus dissociated (in cold PBS pH 7.4) with 80 µL low melting point agarose (0.75%). The

mixture (cells/agarose) was added to a microscope slide coated with a layer of 500 µL of normal melting agarose (1%). After solidification, the cover slip was gently removed and the slides were placed overnight in lysis solution (2.25 M NaCl, 90 mM EDTA and 9 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% dimethylsulfoxide [DMSO]). Subsequently, the slides were incubated in freshly prepared alkaline solution (300 mM NaOH and 1 mM EDTA, pH 12.6) for 5 min. The electrophoresis of DNA was performed for 20 min at 25 V (0.90 V/cm) and 300 mA under alkaline conditions (pH >13). After that, the slides were neutralized with 0.4 M Tris (pH 7.5) three times during five minutes. Finally, the DNA was stained with Syber Green. The stained nuclei were blindly analyzed by fluorescence microscopy with visual inspection (200X). Cells were scored from zero (no fragmentation observed) to 4 (maximal fragmentation index), according to the tail intensity (size and shape), resulting in a single DNA fragmentation score for each cell, and consequently, for each group. The DNA fragmentation index was calculated by multiplying the number of cells by its respective index score and then summing it up. Therefore, a group index could range from zero (all cells with no tail, 100 cells X 0) to 400 (all cells with maximally long tails, 100 cells X 4) (Collins et al., 1997).

2.7 Mitochondrial mass and membrane potential measurement

MitoTracker was used for mitochondrial function analysis in cell suspensions of hippocampus obtained by mechanical dissociation with PBS containing collagenase to a density of about 200.000 cells/mL. The dissociated contents were then filtered into a sterile 50 mL Falcon tubes (BD Biosciences) through 40 µm nylon cell strainer (Cell Filter Strainer – BD Biosciences) and kept on ice until mitochondrial staining. To, MitoTracker Red (MTR or Chloromethyl-X-rosamine) and MitoTracker Green (MTG)

dyes were used to assess mitochondrial potential (Δψ) and mass, respectively. MTR and MTG were dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock concentration. Samples as described earlier were stained with 100 nM MTR and 100 nM MTG for 45 min at 37ºC in a water bath in a dark room, according to the method described by Weis et al. (2012). Immediately after staining, cell suspensions were removed from the water bath and analyzed by flow cytometry. Samples stained with MTR and MTG dyes were analyzed on the FACSCalibur and data collected by CellQuest Pro software (Becton Dickinson, San Jose, CA). MitoTracker dyes were excited using 488 air-cooled argon laser. Negative controls (samples without stain) were included for setting up the machine voltages. Controls stained with a single dye were also employed to allow the setting of compensation. The emission of fluorochromes was recorded through detectors and specific band-pass fluorescence filters: red (FL3; 670nm long pass) and green (FL1; 530nm/30). Fluorescence emissions were collected using logarithmic amplification. Briefly, data from 10.000 events (intact cells) were acquired and mean relative fluorescence intensity was determined after exclusion of debris events. All flow cytometric acquisitions and analyses were performed using CELLQuest Pro data acquisition (BD Biosciences) and FlowJo analysis software respectively. Data were analyzed and plotted by density as a single-parameter histogram that shows the relative fluorescence on the x-axis and the number of events (cell count) on the y-axis. The histograms were divided in two halves (named cells with low and high mass or potential), based on the peak of the controls for MTG and MTR, and this evaluation was extended to all data in each parameter. Using this method, analyses of cells resulted in two populations, with different mitochondrial mass and Δψ. The first population presented low mass or Δψ and the second, high mass or Δψ. The lower accumulation of

MTG or MTR, and thus lower fluorescence values would be indicative of decreased mitochondrial mass or $\Delta\psi$ (Khanal et al., 2001; Rodriguez-Enriquez et al., 2009).

2.8 Respiratory chain activity determination

Brain structures were freshly homogenized with a teflon–glass homogenizer (1:20, w/v) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base), pH 7.4, for determination of respiratory chain complexes activities. The homogenates were centrifuged at 1000 g for 10 min at 4°C and the supernatants were immediately kept at -70°C until analyses. Mitochondrial energy metabolism was evaluated using enzymatic analysis of the electron transport chain (ETC) activities. The activities of the ETC complexes I–III, II and IV were determined in homogenates according to standard methods previously described in the literature (Fischer et al., 1985; Rustin et al., 1994; Schapira et al., 1990). The activity of complex I–III (complex I + CoQ + III) was assessed by measuring the increase in absorbance due to cytochrome *c* reduction at 550 nm according to the method described by Schapira et al. (1990). The reaction mixture contained 5 to 10 µg of protein and 20 mM potassium phosphate buffer with 2 mM KCN, 10 mM EDTA and 50 mM cytochrome c, pH 8.0. The reaction was initiated by adding 25 mM NADH and was monitored at 25°C for 3 min before addition of 10 mM rotenone, after which the activity was measured for additional 3 min. Complex I–III activity was the rotenone sensitive NADH: cytochrome *c* reductase activity. The activity of complex II (succinate: 2,6-dichlorophenolindophenol [DCIP] oxiredutase) was determined according to Fischer et al. (1985), following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm. The reaction medium consisting of 40 mM potassium phosphate buffer, pH 7.4, 16.0 mM sodium succinate and 8 µM DCIP was preincubated with 30–60 µg protein at 30 °C for 20 min. After that, 5 mM sodium azide,

8 μM rotenone and 50 μM DCIP were added to the medium and monitored for 5 min. Cytochrome *c* oxidase (COX, complex IV) activity was determined according to Rustin et al. (1994), following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm. The reaction was initiated adding 0.175mg reduced cytochrome *c* in a medium containing 10 mM potassium phosphate buffer, 0.6 mM n-dodecyl-β-D-maltoside, pH 7.0 and 1.5 to 3 μg protein. The activity of complex IV was measured at 25ºC for 10 min. The activity of respiratory chain complexes were calculated and expressed as nmol per min per mg of protein.

2.9 Live cells, Early Apoptosis, Late Apoptosis and Necrosis

To analyze these parameters, cell suspensions of hippocampus (200.000 cells/mL) obtained by mechanical dissociation with PBS containing collagenase were used. The dissociated contents were then filtered into a sterile 50 mL Falcon tube (BD Biosciences) through 40 μm nylon cell strainer (Cell Filter Strainer – BD Biosciences) and kept on ice until cells staining. The live/dead cell assay was performed as described in the kit datasheet (Apoptotic, Necrotic & Health Cells Qualification kit-Uniscience). To assess apoptosis (early apoptosis), dead cells by apoptosis (late apoptosis), necrosis and live cells specific fluorescent probes (annexin V and ethidium homodimer) were employed. Annexin V (FITC labeled) can identify apoptotic cells by binding to phosphatidyl-serine (PS) exposed on the outer leaflet of the plasma membrane that occurs during apoptosis. Ethidium homodimer (EH) is a membrane-impermeable red fluorescent dye which binds to DNA and can be used to detect dead cells by necrosis. Briefly, 10^6 cells were incubated at room temperature with annexin V, EH and binding buffer for 15 minutes. The levels of annexin incorporation and levels of EH positive cells were determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin

Lakes, NJ, USA). FITC and EH dyes were excited at 488 nm. Negative controls (samples without label) were included for setting up the machine voltages. Controls stained with a single dye (FITC and EH) were used to set compensation. The emission of fluorochromes was recorded through detectors using specific band-pass fluorescence filters: green (FL1; 530 nm/30) and red (FL3; 670 nm long pass), and collected using logarithmic amplification and the procedures for acquisition and analyses were similar to those described above for mitochondrial mass and potential determinations. Data were analyzed and plotted by density as a dot plot which shows the relative FL1 fluorescence on the y-axis and the relative FL3 fluorescence on the x-axis (Figure 1). The quadrants to determinate the negative and positive area were placed based on control samples with 80-90% live cells and this setting was applied to all samples. The number of cells in each quadrant was computed and the proportion of negative stained cells or cells stained with annexin, EH or both were expressed as live, apoptotic, necrotic or cells in late apoptosis, respectively (Boersma et al., 1996; Homburg et al., 1995; Martin et al., 1995; Vermes et al., 1995).

2.10 Hippocampal BDNF Assay

Hippocampal BDNF levels were measured by sandwich enzyme-linked immunosorbent assay, using a commercial kit (BDNF E_{max}® Immunoassay system, Promega, USA). Briefly, hippocampi were homogenized 1:10 in a lysis buffer containing 137mM NaCl, 2.5M KCl, 10mM HEPES, 0.6mm EDTA pH7.9, 1%SDS, 10% glycerol and 1% protease inhibitor cocktail (PIC). Microtiter plates (96-well, flat-bottomed) were coated for 24h with the samples, or with standards diluted in sample diluent, and ranged from 7.8 to 500 pg of BDNF. Sequential processing of the samples was performed according to manufacturer instructions.

2.11 Immunoblotting to glucocorticoid receptor (GR)

Hippocampus was homogenized in ice-cold lysis buffer pH 7.9: 2.5M KCl, 10mM HEPES, 0.6mM EDTA, 0.5 %SDS and 1% protease inhibitor cocktail (PIC). Equal protein concentrations (50 µg/lane of total protein) were loaded onto 10% polyacrylamide gels, analyzed by SDS-PAGE and transferred (MiniVE Electrophoresis System Amersham Biosciences) to nitrocellulose membranes (1 h at 25V in transfer buffer [48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS]) (Valentin et al., 2001). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% bovine serum albumin). After incubation, the blot was incubated overnight at 4 °C in blocking solution containing one of the following antibodies: anti-glucocorticoid receptor (GR, 1:200; Santa Cruz Biotechnology), anti-β-actin (1:1000, Cell Signaling Technology). The blot was then washed three times for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-rabbit IgG (1:1000, Millipore). The blot was again washed four times for 5 min with T-TBS and then left in TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). The chemiluminescence was detected using X-ray films that were scanned and analyzed using the Image J Software.

2.12 Protein Assay

The protein concentration was determined in the samples using the method described by Lowry et al. (1951) or by Lowry modified according to Peterson et al. (1979), with bovine serum albumin as the standard.

2.13 Statistical Analysis

Data were expressed as means ± standard error of the mean, and were analyzed using Repeated Measures ANOVA for learning in the water-maze task (performance during the training session) and Kruskal-Wallis test for the latency to reach the platform in the test session. For all other evaluations, a two-way ANOVA (factors: stress and diet) was used. Significance level was accepted as different when the *P* value was less than 0.05. Regarding Repeated Measures ANOVA, Greenhouse-Greisser correction was applied considering violation of the sphericity assumption as shown by the Mauchly test. Post-hoc tests were performed and indicated in the Results when estatistical significance was reached.

3. Results

3.1 Performance in the water maze task

During the learning phase of the task (Figure 1A), there was a significant interaction between time x stress x diet [$F(5.58, 279.48) = 2.44$, $P = 0.028$, repeated measures ANOVA, correction for Greenhouse-Geisser], since the latency to find the platform decreased over time for all groups; however, the group exposed to isolation stress and with access to a palatable diet showed a smaller decrease in latency during the repeated sessions. During the test session, the latency to find the original place of the platform (Kruskal-Wallis test, $P > 0.05$; see Figure 1B) was similar for all the groups; the number of crossings on the original place of the platform and the time spent to target and in the opposite quadrants (two-way ANOVA, $P > 0.05$ in both cases, see Table 1) were also similar for all groups. Additionally, we observed that both, the exposure to isolation stress during the prepubertal period [$F(1,42) = 14.44$, $P < 0.001$], and the palatable diet [$F(1,42) = 6.46$, $P = 0.014$], reduced swimming speed (Table 1).

3.2 Crossings in the open field

Data of open field are shown in Figure 2. There was a significant interaction between stress and diet on the number of crossings during the first session (first exposure to this environment) [F(1,35) = 7.58, P <0.01], since both, isolation stress and palatable diet, had a tendency to decrease this number. However, when they were applied together, the number of crossings was similar to the control group. On the other hand, in the second exposure to the same environment, there was no differences on the number of crossings between groups (P>0.05), indicating that the isolation stress and palatable diet intake in the early life do not lead to significant impairment in motor activity in adulthood.

3.3 Antioxidant enzyme activities and free radicals production

To verify whether exposure to isolation stress and palatable diet in the prepubertal period induce a long-term oxidative imbalance in hippocampus of rats, we analyzed the antioxidant enzyme activities and free radicals production. The evaluation of SOD activity (Figure 3A) at 60 days (adult rats), showed an interaction [*F* (1,16)= 5.02, P< 0.005], with a potentiation of SOD activity when both factors, stress and palatable diet, were present together. A post-hoc Tukey test showed that stressed groups are different from control groups (P < 0.05). GPx (Figure 3B) and CAT (Figure 3C) activities and thiol content (Figure 3D) were not altered by either isolation stress or palatable diet (P>0.05). Additionally, only isolation stress increased free radicals production [*F* (1,16)= 6.42, P<0.05], as evaluated through the DCFH test (Figure 3E).

Regarding SOD/CAT and SOD/GPx ratios (Table 2), isolation stress increased SOD/CAT [*F* (1,16)= 28.37, P< 0.001] and SOD/ GPx [*F* (1,15)= 27.83, P< 0.001]

ratios, while the palatable diet only increased SOD/ GPx ratio [F (1,15)= 9.96, P< 0.01]. A post-hoc Tukey test showed that both groups subjected to isolation stress had increased ratios than non-stressed groups (P < 0.05).

3.4 *DNA* fragmentation index

A significant interaction was observed in DNA fragmentation index in the hippocampus of adult male rats [F (1,14)= 22.80, P< 0.001]. This was due to the fact that either palatable diet or exposure to isolation stress increased DNA fragmentation index [F (1,14) = 15.53, P < 0.002; F (1,14)= 7.72, P< 0.05, respectively]. However, when both, isolation stress exposure and palatable diet were present, the index was similar to controls. A post-hoc Tukey indicated that control group receiving diet was different from every other group (P < 0.05).

3.5 Mitochondrial mass and membrane potential

Analysis of hippocampal cells labeled with MTG and MTR are shown in Figures 5 and 6, respectively. We observed a significant interaction between palatable diet and stress exposure [F (1,16)= 14.64, *P<0.01*], since the group subjected to isolation stress and receiving palatable diet showed increased number of cells with low mitochondrial mass (Figure 5A). A significant interaction between these factors was also observed for cells with high mitochondrial mass [F (1,16)= 16.91, *P<0.001*], since the group subjected to both isolation stress and palatable diet showed reduced number of cells with high mitochondrial mass (Figure 5B). There were no effects on cell with low $\Delta\psi$ (Figure 6A) in the hippocampus these animals (P>0.05); however, a significant interaction between isolation stress and palatable diet consumption was observed in cells with high mitochondrial potential ($\Delta\psi$) [F (1,16)= 7.95, P<0.05], since the number

of cells with high potential decreased when palatable diet was associated with stress (Figure 6B).

3.6 Respiratory chain enzymes activities

Mitochondrial energy metabolism in the hippocampus was evaluated using enzymatic analysis of electron transport chain (ETC) activities (Table 3). There was an interaction stress x diet (two-way ANOVA, [F (1,11)= 4.92, P<0.05] on Complex II activity, since when both factors are together, activity returned to levels similar to the control group. In addition, there was an interaction stress x diet (two-way ANOVA, [F (1,12)= 6.31, P<0.05] on Complex IV activity, since stress exposure increased this activity; however, when both factors are together, activity returned to values similar to the control group. No significant difference was observed in the activity of complex I+III (P>0.05).

3.7 Flow cytometry analysis after annexin V and ethidium homodimer staining

As showed in Figure 7, the exposure to a palatable diet decreased the number of live cells [F (1,15)=4.35, $P<0.05$] (Figure 7A), and induced an increase in the number of positive cells labeled with annexin-V (early apoptosis; Figure 7B) [F (1,15)=7.27, $P<0.02$]. The isolation stress decreased the number of positive cells double labeling (late apoptosis; Figure 7C) [F (1,15)=6.76, $P<0.05$].

Regarding the necrosis, a statistical significant interaction was observed between stress and palatable diet [F (1,15)=13.06, $P<0.01$] (Figure 7D), since both isolated factors increased necrosis, but the number of cells stained with EH returned to control levels in animals that received palatable diet and were exposed to stress.

3.8 Hippocampal BDNF

There was no significant difference in the hippocampal BDNF levels of adult male rats exposed to isolation stress and palatable diet in the prepubertal period (P>0.05) (Table 4).

3.9 Glucocorticoid receptor (GR)

We observed that the intake of palatable diet in the prepubertal period increased the GR immunocontent in the hippocampus of adult rats (two-way ANOVA [F (1,13)=4.70, $P<0.05$]) (Figure 8).

4. Discussion

Previous studies from our laboratory have demonstrated a high vulnerability of juvenile male rat hippocampus when the animals were exposed to isolation stress during the prepubertal period (Krolow et al., 2013). Additionally, free access to a palatable diet was found to prevent some of the stress-induced effects in the hippocampus of these juvenile animals (Krolow et al., 2013). Based on these findings, here we investigated whether these effects could be long-lasting and whether these neurochemical changes would affect the spatial memory of the animals. Table 5 shows a summary of statistical significant effects of stress, diet or the interaction.

During the learning phase of the water maze task, the group that was subjected to isolation stress while receiving palatable diet showed impairment on the repeated sessions. At the test day, however, no difference between groups was observed, and all groups exhibited adequate spatial memory. However, the stress and palatable diet groups reduced the swimming speed. Thus, this result suggests that the

effect on the latency to reach the platform during the learning phase may not be attributed to a cognitive impairment, since memory was not altered in the test session. The reduced latency may be related to the reduced swimming speed of animals exposed to both isolation stress and palatable diet.

Additionally, during the first exposure to the open field apparatus (novel environment), both stressed and palatable diet groups had a tendency to decrease the number of crossings. However, the group exposed to stress and palatable diet together exhibited performance similar to controls. In the next session (familiar environment) there were no differences between groups, indicating that the isolation stress and palatable diet during the early life did not lead to significant changes in motor activity in adulthood. Although some studies using prolonged social isolation have reported increased locomotor activity (Levine et al., 2007), stress during the prepubertal period, as used in this study, was not able to produce the complete behavioral changes known to result from long periods of isolation (Fulford and Marsden, 2007; Lapiz et al., 2001). Therefore, the effects observed in the water maze may not be related to motor activity or cognition, but to other factors, such as emotionality or motivation. Considering the reduced swimming speed, it is possible that animals stressed by social isolation receiving palatable diet in the prepubertal period are less motivated to find the platform. However, to confirm such hypothesis further studies should be performed.

Lifestyle factors, including exercise, diet, and stress exposure are increasingly recognized as determinants of adaptive or maladaptive hippocampal functioning (Magarinõs and McEwen, 1995; Van Praag et al., 2005), and they have been linked to levels of BDNF. The BDNF is a neurotrophin involved in synaptic plasticity and memory processes (Poo, 2001; Tyler et al., 2002), including spatial learning

(Linnarsson et al., 1997; Mizuno et al., 2000) and memory formation (Mizuno et al., 2000). Although BDNF seems to be altered in several psychiatric conditions, including depressive states (Martinowich et al., 2007), we were unable to detect any difference in BDNF levels in hippocampus for all groups.

In order to study if the neurochemical changes induced by stress during the pre-pubertal period (Krolow et al., 2013) were long-lasting, we evaluated several parameters related to cellular endangerment in the hippocampus of adult male rats exposed to isolation stress during the pre-pubertal period, when receiving or not palatable diet. Regarding respiratory complex activities, we observed that exposure to stress increased activity of Complex IV. It has been suggested that the expression of genes that code for cytochrome oxidase (responsible for the catalytic activity of Complex IV) is induced by glucocorticoids (Serviddio et al., 2011). These hormones are also known to increase oxidative stress, leading to neural endangerment by the increased generation of ROS (McIntosh and Sapolsky, 1996, Madrigal et al., 2001), which can directly damage lipids, nucleic acids and proteins (Blumberg, 2004; Evans et al., 2004). Accordingly, previous data from our group demonstrated that exposure to stress during the prepubertal period induces hippocampal oxidative imbalance in juvenile rats (Krolow et al., 2013). Here, we found that isolation stress had long-lasting effects on free radicals production, increasing ROS, and SOD/CAT and SOD/GPx ratios. The increased SOD and reduced CAT and GPx activities, may result in higher concentration of hydrogen peroxide (H_2O_2). The SOD/CAT and SOD/GPx ratios have been used as indicators of peroxide overload challenge (De Oliveira et al., 2007; Pinho et al., 2006), where SOD converts superoxide to H_2O_2, but CAT and GPx are not able to metabolize it efficiently. The production of both, superoxide and H_2O_2 has been reported to be enhanced after stress (Ward and Till, 1990). Therefore, the oxidative

imbalance observed in hippocampus from juveniles exposed to isolation stress remains present in the adult brain long after the cessation of the isolation period, making this structure more vulnerable to injury by a number of factors, and this situation could be related to the increased number of necrotic cells observed.

Exposure to stressful events leads to an increased release of GCs through the activation of the HPA axis. Glucocorticoid receptors (GR) are located in distinct brain structures, mainly in the hippocampus (McEwen, 2008; Sapolsky, 2003), and the high vulnerability of hippocampus to ROS, as reported above, appears to be related to the abundant expression of GR (Sato et al., 2010). In the hippocampus, GR are also important to the control of HPA axis by negative feedback (McEwen, 2008; Sapolsky, 2003). Therefore, we analyzed the immunocontent of hipocampal GR. Our result showed that the palatable diet increased the content of GR in hippocampus of adult male rats. This increase in the GRs could make the HPA axis more responsive to the negative feedback, what would reduce the stress response. Some studies showed that the regulation of the HPA axis depend on the type of palatable food taken (Kamara et al., 1998; Pecoraro et al., 2004; Tannenbaum et al., 1997). In adult animals, diets rich in calories and sugar are suggested to reduce the axis response to stress (Pecoraro et al., 2004), acting on the negative feedback of GCs, and this was in agreement with our results on GR levels in hippocampus. This effect of the intake of a palatable diet on GR levels in hippocampus accentuates the negative feedback; bring more efficiently the GCs levels to normal values after the stress response, reducing some of the stress consequences on these animals, and could help to explain the interactions found in the present study. Several parameters were affected by exposure to stress or diet and were returned to normal, when both factors were applied simultaneously, including the

activities of respiratory chain Complexes II and IV, DNA fragmentation index, number of cells in late apoptosis and in necrosis stages.

On the other hand, we found that the palatable diet had some long lasting effects *per se*. A previous study showed that chronic intake of palatable diet caused an imbalance on antioxidant enzymes in hippocampus of adult rats (Krolow et al. 2010). Here, we also observed increased SOD/GPx ratio. This imbalance may lead to higher concentration of H_2O_2, and this excess of H_2O_2 facilitates the production of hydroxyl radical ($OH^{\cdot}$), the most powerful oxidant molecule that reacts with iron or copper (Fenton chemistry) (Halliwell, 2006). When oxidative stress occur, it may result in damage of biomolecules, including DNA (Lovell and Markesbery, 2007; Winyard et al., 2005). DNA strand breakage can be induced by nucleases activated by Ca^{2+} and/or ROS, mainly $OH^{\bullet}$ formed by reaction of H_2O_2 with DNA-bound metal ions (Darley-Usmar and Halliwell, 1996). Additionally, some studies have presented evidence that excessive intake of palatable foods can lead to fragmentation in cellular DNA (Krolow et al., 2010; Olivo-Marston et al., 2008). Our results showed that palatable diet in the prepubertal period increased the DNA fragmentation index in adulthood. Interestingly, when the stress and palatable diet were applied together, DNA fragmentation levels returned to normal. Accordingly, previous studies suggested that the palatable food intake, rich in fat and carbohydrates, may decrease the stress response in chronically-stressed rats (Fachin et al., 2008; Pecoraro et al., 2004), as discussed above.

Oxidative stress-induced cellular damage is considered a central event in apoptosis (Franklin, 2011; Niizuma et al., 2010; Valko et al., 2006). In the present study, we measured the number of apoptotic and necrotic cells. The exposure to palatable diet induced a decrease in the number of live cells and an increase in the

number of cells in early apoptosis. Therefore, the intake of palatable diet during the prepubertal period appears to lead to neurochemical changes in the hippocampus, which may be maladaptive, especially concerning susceptibility to apoptosis.

The access to a palatable diet during the period of exposure to isolation stress was able to prevent some long-term effects of stress, as discussed above. However, we also observed that some other parameters appear to be further impaired by the association of these two factors. Hippocampal cells from stressed animals that received palatable diet had higher SOD activity, which could suggest a reaction of these cells to increased production of superoxide (Halliwell, 2006). In addition, they also presented lower mitochondrial mass and potential, suggesting reduced mitochondrial biogenesis. Long-term intakes of high fat diets by adult rats have also shown to induce mitochondrial damage (Du et al., 2012). To our knowledge, this is the first report showing that a diet rich in sugars during development may also lead to mitochondrial dysfunction in the adulthood. Concerning stress exposure, we had previously observed that isolation stress increase mitochondrial potential in the hippocampus of juvenile rats (Krolow et al. 2013). Therefore, this reduced mitochondrial function in stressed animals that received palatable diet is a long- term effect, and was not present soon after stress exposure. However, at this point, we cannot determine if the general effects of palatable diet and isolation stress on mitochondrial function would be either adaptive or maladaptive.

In conclusion, isolation stress during the prepubertal period did not affect spatial memory, but, when applied together with a palatable diet, reduced swimming speed, that may be related to lower motivation. This early stress exposure also induced long-lasting changes in the hippocampal oxidative balance (increasing reactive species

generation and SOD:CAT and SOD:GPx ratios). The intake of palatable diet during the stress exposure attenuated some of these effects (such as respiratory chain enzymes activities, DNA fragmentation index and number of necrotic cells), which could be related to the increased GR in this structure. However, it also led to some possibly maladaptive effects on behavior and on mitochondrial biogenesis. Therefore, we suggest that the behavioral changes observed are related to motivation rather than cognitive aspects. Since mitochondrial dysfunctions are known to be involved with mood disorders (Saradol et al., 2007), it is possible that these behavioral changes result from modification in the neurochemical parameters evaluated. Thus, stress and palatable diet during the prepubertal period may increase vulnerability during adulthood and understanding their effects may enable us to prevent chronic conditions in the adult.

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Disclosure statement

This paper does not present conflict of interest.

Authors' contributions

Krolow R participated in the implementation and supervision of all experiments; Noschang C; Arcego DM; Marcolin ML; participated in developing the methodology, especially with the accompanying of the isolation stress and consumption of diet palatable; Noschang C , Toniazzo AP, Lazzaretti C, Weis SN, Pettenuzzo LF, Ferreira

C, and Benitz AN, participated in behavioral or biochemical experiments. Dalmaz C directed and oversaw all development of this work.

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Table 1. Effects of isolation stress and consumption of palatable diet during the prepubertal period on performance of adult rats in the water maze task during the test session. Number of crossings (on the original place of the platform), time spent in target and opposite quadrants (s), and swimming speed (average speed in m traveled during 60 s) are expressed as mean ± SEM . N = 12-15/group.

Group	*Number of Crossings*	*Time in target quadrant (s)*	*Time in opposite quadrant (s)*	*Swimming speed (m/min)*
Control + Chow	1.93 ± 0.37	24.42± 2.10	7.35± 1.85	0.23 ± 0.013
Control + Palatable Diet	2.07 ± 0.56	23.38± 3.02	6.46± 1.95	0.21 ± 0.018
Stressed + Chow	2.33 ± 0.43	24.66± 2.38	5.46± 1.20	0.20 ± 0.013
Stressed + Palatable Diet	1.16 ± 0.38	21.16± 5.00	4.75± 5.1.61	0.14 ± 0.02*

Stress and palatable diet decreased swimming speed (Two-way Anova, $P<0.001$ and $P=0.014$, respectively).

* Significant difference from all other groups (Post hoc Tukey test, $P < 0.05$).

Table 2. Effects of isolation stress and consumption of a palatable diet during the prepubertal period on *SOD/GPx* and *SOD/CAT* ratios in the hippocampus of adult rats. Data are expressed as mean ± SEM . N = 5/group.

Group	*SOD/GPx* ratio	*SOD/CAT* ratio
Control + Chow	0.32 ± 0.03	4.13 ± 0.68
Control + Palatable Diet	0.52 ± 0.13	4.07 ± 0.56
Stressed + Chow	0.71 ± 0.08*	7.83 ± 0.95*
Stressed + Palatable Diet	1.11 ± 0.09*	10.59 ± 1.40*

Stress increased SOD/CAT and SOD/GPx ratios (Two-way Anova, $P<0.001$). Palatable diet increased SOD/GPx ratio (Two-way Anova, $P<0.001$).

* Significant difference from non-stressed groups (Post hoc Tukey test, $P < 0.05$).

Table 3. Effects of isolation stress and consumption of palatable diet during the prepubertal period on respiratory chain enzyme activities (nmol/min/mg protein) in the hippocampus of adult rats. Data are expressed as mean ± SEM of cytochrome c reduction (Complex I + III), 2,6-DCIP reduction (Complex II) and oxidation of previously reduced cytochrome c (Complex IV). N =4-5/group.

Group	Complex I-III	Complex II	Complex IV
Control + Chow	2.72 ± 0.65	5.85 ± 0.32	139.2 ± 8.29
Control + Palatable Diet	2.46 ± 0.38	4.70 ± 0.50	136.2 ± 9.39
Stressed + Chow	4.12 ± 0.41	3.83 ± 0.63	186.0 ± 21.49*
Stressed + Palatable Diet	2.49 ± 0.16	5.15 ± 0.80❖	117±2.64❖

❖ There was an interaction stress x diet on Complex II and on Complex IV activities (two-way ANOVA, $P < 0.05$).

* Significant difference from stressed group receiving palatable diet (Post hoc Tukey test, $P < 0.05$).

Table 4. Effects of isolation stress and consumption of palatable diet during the pre-pubertal period on hippocampal BDNF levels (pg of BDNF/ mg protein) in adult male rats. Data are expressed as mean ± SEM . N = 6/group.

Group	*BDNF levels*
Control + Chow	18.81 ± 1.19
Control + Palatable Diet	19.13 ± 0.48
Stressed + Chow	19.29± 0.91
Stressed + Palatable Diet	22.89 ± 0.99

There was no significant difference between groups (Two-way- Anova, $P > 0.05$).

Table 5. Summary table of the effects of isolation stress and consumption of palatable diet during the pre-pubertal period in adult male rats. Only statistical significant effects are shown.

Parameter evaluated		Isolation stress effect	Palatable diet effect	Interaction stress x diet
Behavioral evaluation	Learning curve in the Morris maze			↓
	Swimming speed	↓	↓	
	Ambulation			Return to control levels
Neurochemical evaluation in hippocampus	ROS production	↑		
	SOD activity			↑
	SOD:CAT ratio	↑		
	SOD:GPx ratio	↑	↑	
	DNA fragmentation index		↑	Return to control levels
	Mitochondrial mass			↓
	Mitochondrial potential			↓
	Mitochondrial Complex II activity			Return to control levels
	Mitochondrial Complex IV activity	↑		Return to control levels
	Glucocorticoid receptors		↑	
	Number o live cells		↓	
	Number of necrotic cells	↑		Return to control levels
	Number of cells in early apoptosis		↑	
	Number of cells in late apoptosis			↓

Legends to figures

Figure1: Effects of isolation stress and consumption of palatable diet on performance in the water maze task in adult rats. N=12-15/group. **A**. Performance during training . Data are expressed as mean ± S.E.M. (s); **B.** Latency to find the platform in the test session(s). Data are expressed as median (interquartile range).

❖Interaction between stress x palatable diet x session (correction for Greenhouse-Geisser P =0.028).

Figure 2: Effects of isolation stress and consumption of palatable diet on crossings in the open field (in two sessions) in adult rats. Data are expressed as mean + S.E.M. N=9-10/group.

❖Interaction between stress x palatable diet (Two-way ANOVA, $P<0.01$) in the first exposure.

Figure 3: Effects of isolation stress and consumption of palatable diet on antioxidant enzyme activities, free radical (DCFH test) production and on total thiol levels in the hippocampus of adult rats. Data are expressed as mean + S.E.M. N=4-5/group. **A** SOD (expressed as U/mg protein), **B** GPx (expressed as nmol NADPH consumed/min/ mg protein), **C** CAT (expressed as micromoles of H_2O_2 consumed/min/mg protein), **D** total thiols (expressed as nmol TNB/mg protein) and **E** DCFH (expressed as nmol of DCF formed/mg protein).

❖Interaction between stress x palatable diet on SOD activity (Two-way ANOVA, $P<0.005$).

*Stressed groups are different from non-stressed groups (post-hoc Tukey test, $P < 0.05$)

Stress increased free radicals production (Two-way ANOVA, P<0.05).

Figure 4. Effect of isolation stress and consumption of palatable diet during the prepubertal period on comet assay (expressed as DNA fragmentation index) in the hippocampus of adult rats. Data are expressed as mean + S.E.M. N=5/group.

❖Interaction between stress x palatable diet decreasing DNA fragmentation index (Two-way ANOVA, P<0.001).

* Significantly different from the other groups (post-hoc Tukey test, P < 0.05).

Figure 5. Effects of isolation stress and consumption of palatable diet on mitochondrial mass (cells labeled with Mitotracker Green) in the hippocampus of adult rats. Data are expressed as mean ± S.E.M. N=4-5/group. **A** Low Mass, **B** High Mass.

❖ Interaction between stress and palatable diet reduced mitochondrial mass (lower number of cells with high mass and increased number of cells with low mitochondrial mass; Two-way ANOVA, P<0.01).

* Significantly different from the group receiving standard chow (post-hoc Tukey test, P < 0.05).

Figure 6. Effects of isolation stress and consumption of palatable diet on mitochondrial potential (cells labeled with Mitotracker Red) in the hippocampus of adult rats. Data are expressed as mean ± S.E.M. N=4-5/group. **A** Low Potential, **B** High Potential.

❖ Interaction between stress x palatable diet decreased cells with high mitochondrial potential, (Two-way Anova, P<0.05).

Figure 7. Effects of isolation stress and consumption of palatable diet on the number of live cells, and on apoptosis, late apoptosis and necrosis in the hippocampus of adult animals. Data are expressed as mean ± S.E.M. N=4-5/group. **A** Live cells (Annexin V^-/EH^-), **B** Early Apoptosis (Annexin V^+/EH^-) **C** Late apoptosis (Annexin V^+/EH^+) and **D** Necrosis (Annexin V^-/EH^+).

Palatable diet decreased the number of live cells (Two-way ANOVA, P=0.05) and increased early apoptosis (Two-way ANOVA, P<0.02).

Stress decreased late apoptosis (Two-way ANOVA, P<0.05).

❖ Interaction between stress x palatable diet decreased necrosis (Two-way ANOVA, P<0.01).

** Significantly different from the other groups (post-hoc Tukey test, $P < 0.05$).

* Significantly different from control group receiving standard chow and from stressed group receiving palatable diet (post-hoc Tukey test, $P < 0.05$).

Figure 8. Effects of isolation stress and consumption of palatable diet on glucocorticoid receptors (GR) content. Data are expressed as mean ± S.E.M. N=4-5/group. **A.** Representative Western Blotting showing imunocontent to GR and β-actin. **B.** Quantification of GR imunocontent by β-actin imunocontent.

Palatable diet increased GR content (Two-way ANOVA, P<0.05).

Figure(s)
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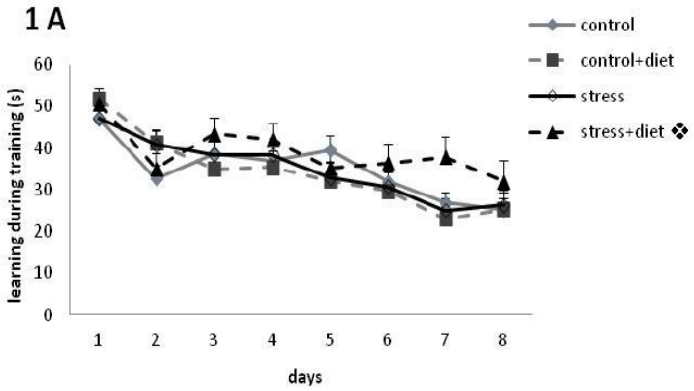

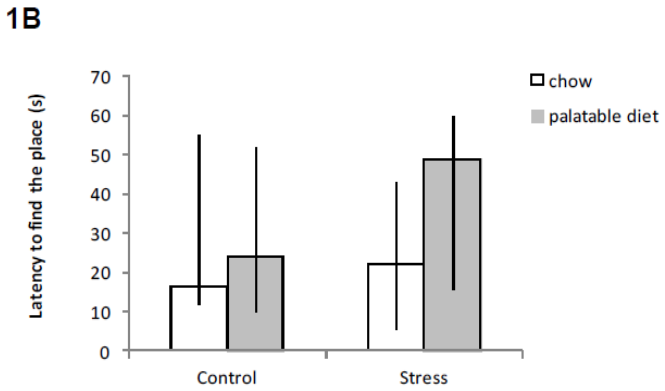

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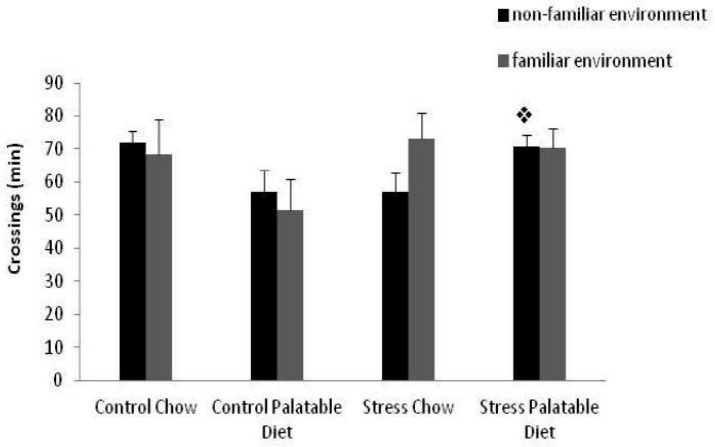

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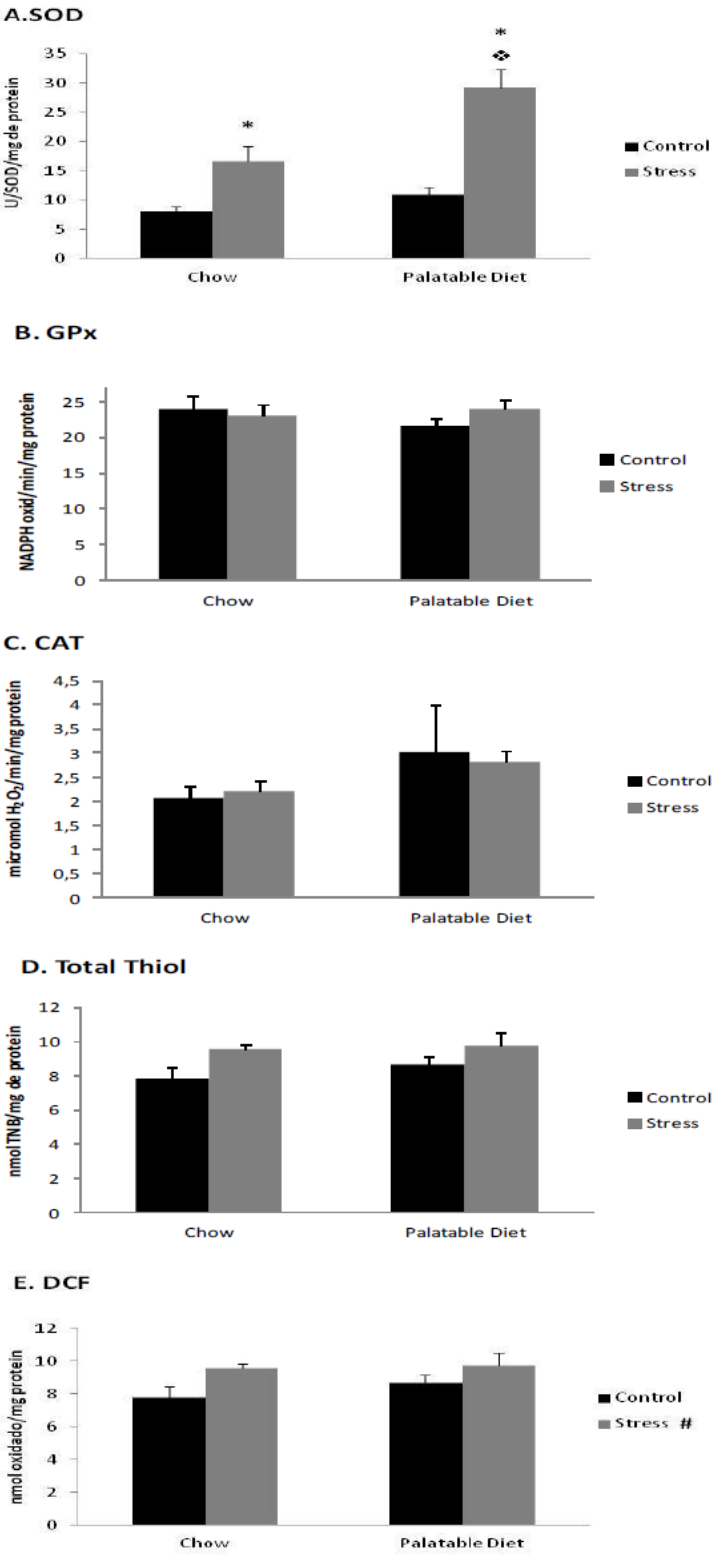

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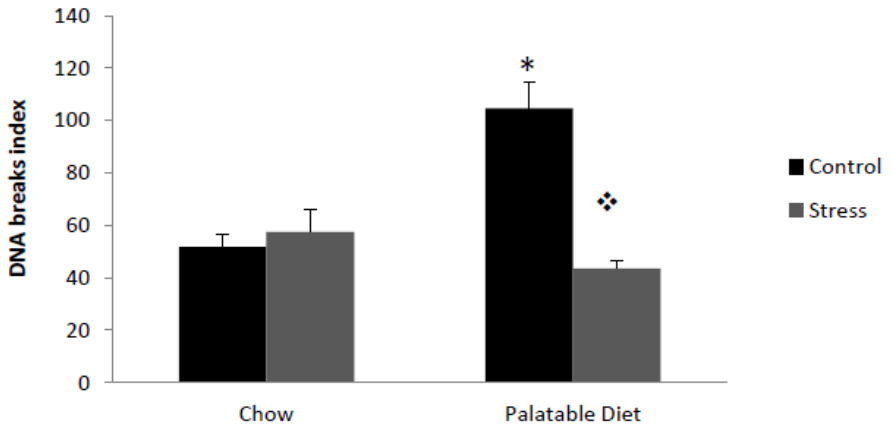

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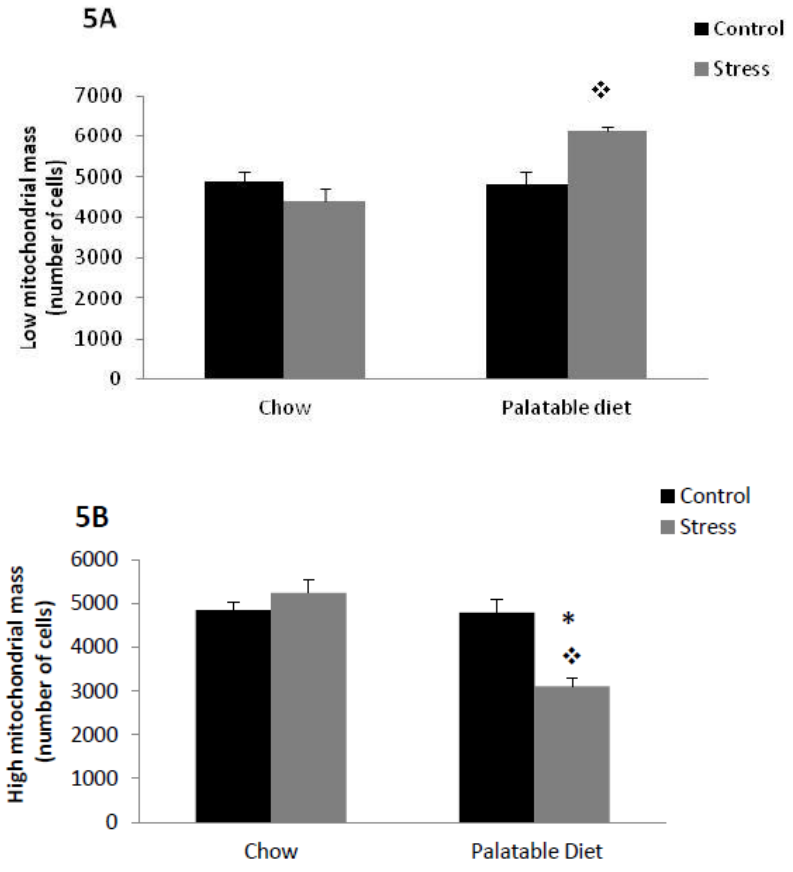

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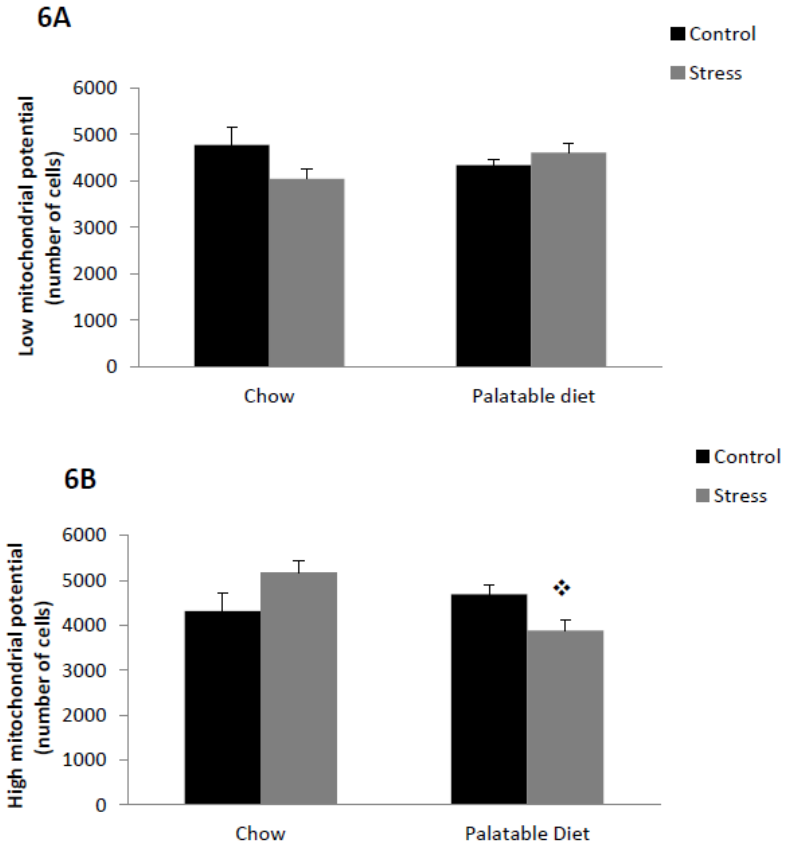

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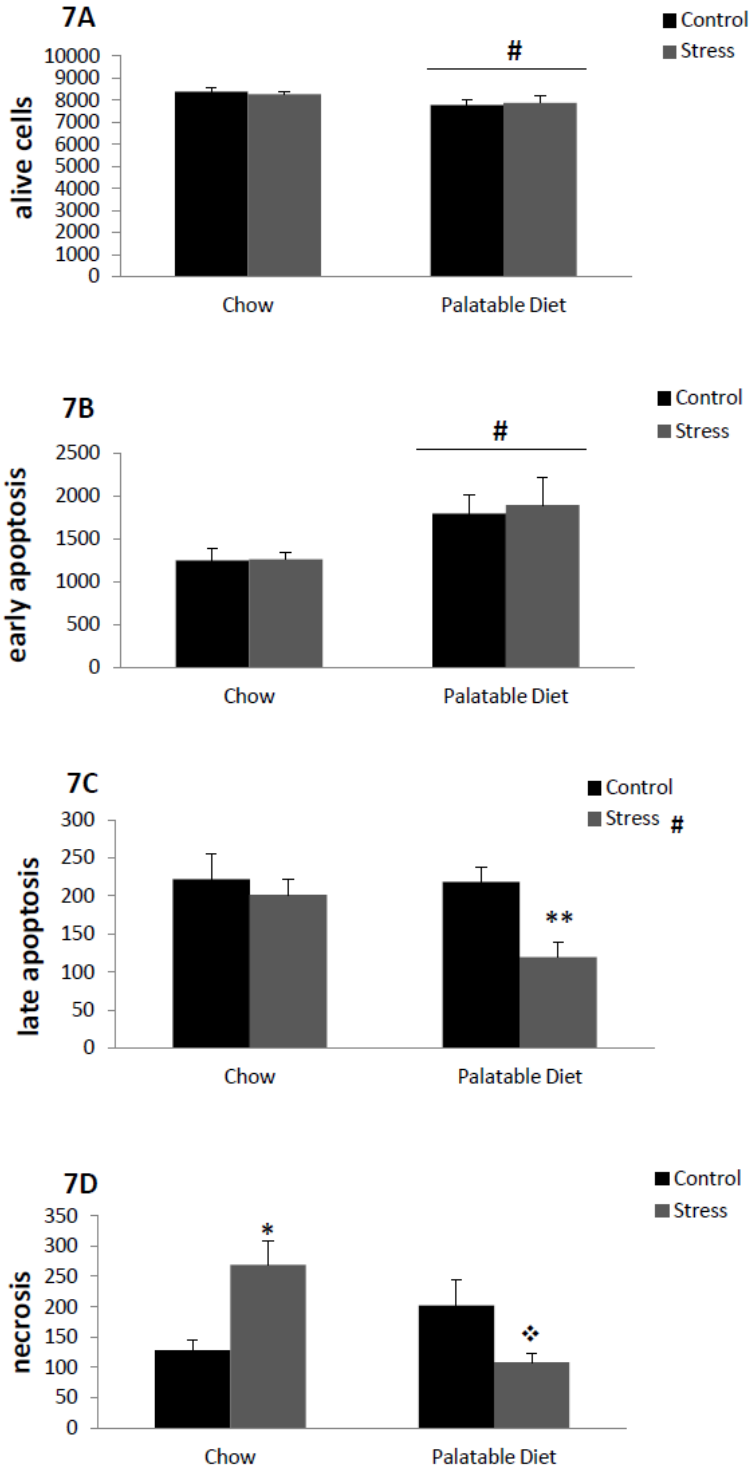

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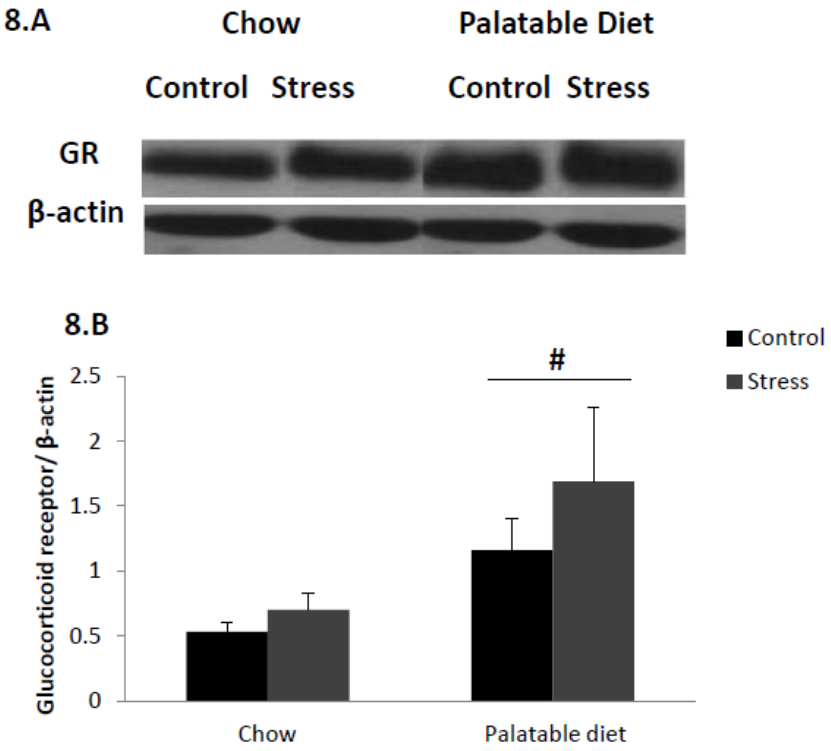

DISCUSSÃO

A presente tese teve por objetivo investigar os efeitos do estresse por isolamento social e do consumo de um alimento palatável no período pré-púbere sobre aspectos metabólicos, neuroquímicos e comportamentais em ratos jovens e adultos. É importante ressaltar que o período em que os animais foram submetidos a esses fatores ambientais, o período pré-púbere, é marcado por constantes alterações no comportamento e na cognição (DAHL, 2004), sendo também marcado por intensa maturação encefálica, incluindo os circuitos que controlam a homeostase energética e as respostas ao estresse (MCCORMICK e MATHEWS, 2007). A influência de fatores ambientais em períodos precoces do desenvolvimento pode levar a alterações persistentes no SNC (MCQUILLEN e FERRIERO, 2004), e no sistema endócrino-metabólico (SCHIMIDT ET AL., 2009). Com base nessas considerações, nossa hipótese era que o estresse por isolamento no período pré-púbere poderia marcar o sistema nervoso de modo a alterar suas respostas fisiológicas, e que uma dieta palatável, em função de suas características confortantes e de suas próprias ações sobre o metabolismo, interagiria com os efeitos do estresse. Desse modo, avaliamos parâmetros relacionados com o metabolismo periférico e também com a função celular neural. Muitos de nossos achados mostraram que essas intervenções precoces podem de certa forma, programar o metabolismo de maneira inequívoca na idade adulta.

Com relação aos principais achados metabólicos periféricos, foi observado que as ratas jovens aumentaram o consumo de dieta palatável quando eram expostas ao isolamento social, o que refletiu-se em aumento no ganho de peso e nos níveis de triglicerídeos plasmáticos. De modo interessante, também foi observado aumento nos níveis plasmáticos de adiponectina. No entanto, muitos destes efeitos observados desapareceram quando os animais submetidos no período pré-púbere ao isolamento social e ao consumo de uma dieta palatável atingiram a idade adulta. Por outro lado,

diferentes efeitos foram encontrados nos ratos machos em diferentes idades. Quando jovens esses animais, submetidos ao estresse por isolamento social aumentaram os níveis plasmáticos de grelina desacetilada e NPY hipotalâmico. Subsequentemente, ratos machos adultos que foram expostos a uma dieta palatável no período pré-púbere mostraram um aumento no peso corporal, no depósito de gordura retroperitoneal e na glicemia. Também foi observada uma diminuição nos níveis plasmáticos de adiponectina e no NPY hipotalâmico. Além desses efeitos, os ratos machos adultos submetidos à exposição ao estresse por isolamento social na pré-puberdade mostraram aumento no peso das adrenais e nos níveis plasmáticos de triglicerídeos, ao mesmo tempo em que se observou uma diminuição no colesterol-LDL.

Esses achados ressaltam as diferenças sexo-específicas relacionados aos efeitos de intervenções durante o desenvolvimento sobre o metabolismo. Aqui é importante ressaltar que esses animais foram submetidos a tais intervenções antes de entrarem na puberdade, de modo que não havia grandes diferenças nos níveis de hormônios gonadais, e mesmo assim responderam diferentemente à influência desses fatores ambientais (estresse e dieta) aos quais foram submetidos. A curva temporal do desenvolvimento sexual dos ratos é conhecida, e sabe-se que, nas fêmeas, por exemplo, o início da ovulação ocorre a partir do dia 29 pós-natal (URBANSKI E OJEDA 1985). Assim, o período juvenil, estudado nesta tese, corresponde ao período em que os órgãos sexuais estão se desenvolvendo e o encéfalo, começa a responder a estímulos de hormônios sexuais (GILLIES E MCARTHUR, 2010). Nas fêmeas, pulsos de LH ocorrem durante essa fase (URBANSKI E OJEDA 1985), mas em níveis insuficientes para causar ovulação, de modo que os folículos tornam-se atrésicos (PICUT ET AL., 2013). Nesse período, os níveis de estrógenos e andrógenos são baixos, mas já detectáveis, sendo que apresentarão um grande aumento após a puberdade (Gillies e

McArthur, 2010). Este fato é interessante, pois o período da puberdade é marcado pela maturação dos circuitos relacionados à produção e liberação de hormônios sexuais, e neste trabalho diferenças entre os sexos com relação ao metabolismo foram percebidas ainda antes da puberdade. As diferenças observadas relacionam-se ao consumo de alimento palatável, ao perfil lipídico e aos hormônios envolvidos na regulação homeostática do comportamento alimentar. Ambos, machos e fêmeas, aumentaram o consumo de uma dieta palatável durante o período pré-púbere, possivelmente devido à palatabilidade desta dieta. Também foi observado que os animais estressados de ambos os sexos aumentaram o consumo deste alimento rico em carboidratos simples. No entanto, as ratas jovens, quando eram expostas ao isolamento social, apresentaram um aumento mais marcante no consumo de uma dieta palatável durante este período do desenvolvimento. Esses achados sugerem que as fêmeas, mesmo antes da puberdade, são mais susceptíveis ao uso de alimentos confortantes como uma forma de reduzir a resposta ao estresse. O uso de alimentos capazes de acionar circuitos de recompensa como um possível mecanismo para reduzir a resposta ao estresse tem sido proposto (PECORARO ET AL., 2005). Além disso, estudos mostram que fêmeas estressadas na idade adulta consomem mais alimentos palatáveis quando esses são oferecidos cronicamente, em comparação com machos (FACHIN ET AL., 2008; ZYLAN, 1996). É importante destacar que a oferta do alimento palatável no presente trabalho, ocorreu entre o vigésimo primeiro e vigésimo oitavo dias de idade pós-natal, quando os animais eram jovens, diferentemente dos outros estudos citados acima, que avaliaram esse parâmetro na idade adulta. Sabe-se também que mulheres são mais susceptíveis a distúrbios do comportamento alimentar que homens (PEBLES ET AL., 2006). Assim, nossos resultados mostram que as ratas fêmeas estressadas em períodos mais precoces da vida já consomem mais alimentos palatáveis e que as diferenças entre os sexos com

relação ao parâmetro estudado (consumo de alimento palatável em situação de estresse social) não dependeram dos níveis circulantes dos hormônios gonadais. Sugerimos então, que a possível maior susceptibilidade de fêmeas a distúrbios do comportamento alimentar é pelo menos em parte, independente de hormônios gonadais circulantes.

Intervenções durante o período pré-púbere podem apresentar efeitos a longo-prazo no consumo alimentar. Os resultados da presente tese mostraram que a dieta palatável oferecida antes da puberdade programou o aumento do consumo calórico de alimento padrão durante o desenvolvimento até a idade adulta, principalmente nos ratos machos. Não houve diferença com relação ao consumo de alimento padrão nas fêmeas, indicando que os machos foram mais susceptíveis à influência do consumo precoce de um alimento rico em carboidratos simples, o qual possivelmente programou um aumento no consumo calórico de alimento para atingir suas necessidades metabólicas. Outros achados tais como aumento do peso corporal, do depósito de gordura retroperitoneal e da glicemia foram reflexos deste aumento no consumo de alimentos ao longo da vida, sugerindo fatores de risco para um futuro desenvolvimento de síndrome metabólica, e neste caso os machos apresentaram maior susceptibilidade aos efeitos a longo-prazo.

Efeitos do estresse sobre o metabolismo dos lipídeos também têm se mostrado sexualmente dimórficos (FARADAY, 2002; MCTERMAN ET AL., 2002; RONCARI e VAN, 1978). O estresse por isolamento social diminuiu o colesterol total e o colesterol-LDL em ratos machos adultos enquanto que nas fêmeas houve um aumento nestes parâmetros. Interessante apontar que os efeitos sexo-específicos da exposição precoce ao estresse sobre o metabolismo do colesterol não apareceram antes da maturidade sexual. É possível que a alta síntese de hormônios esteroides, devido a uma maior

ativação do eixo HHA, possa levar a um maior uso de colesterol-LDL da circulação (HOEKSTRA ET AL., 2010). O aumento na ativação do eixo HHA é deduzido a partir do aumento do peso das adrenais de ratos machos adultos que foram estressados precocemente, sugerindo que o paradigma de estresse usado no início da vida teve um impacto na ativação do eixo HHA na idade adulta. No entanto, as ratas fêmeas não mostraram alteração no peso das adrenais, o que pode sugerir que as fêmeas habituam-se mais facilmente ou são mais resilientes após exposição ao estresse, quando se avalia os efeitos ao longo da vida. Taylor et al. (2000) têm sugerido que a resposta ao estresse envolve outros hormônios, tais como a ocitocina, um neurotransmissor relacionado à formação de vínculos intraespecíficos. Aqueles autores propõem que as fêmeas apresentariam uma resposta do tipo "cuidar e ajudar" que possivelmente poderia envolver a ocitocina (*tend and befriend*), e que aumentaria as chances de sobrevivência das fêmeas e de sua prole, e é possível que essa diferente resposta ao estresse, envolvendo distintos circuitos no sistema nervoso e diferentes mediadores químicos, possa estar relacionada a essa maior resiliência ao estresse a longo-prazo.

No capítulo 1 desta tese, também foram observadas algumas alterações induzidas ou pela exposição ao estresse por isolamento social ou pelo consumo de alimento palatável sobre hormônios ou neurotransmissores envolvidos na regulação homeostática do comportamento alimentar. O isolamento social induziu um aumento no NPY hipotalâmico em ratos machos jovens. Sabe-se que o hipotálamo é um importante centro controlador do comportamento alimentar e também da resposta ao estresse. O aumento no NPY hipotalâmico induzido pela exposição ao estresse pode estar relacionado a um maior estímulo de neurônios orexigênicos produtores deste peptídeo, devido a um aumento marginal nos níveis de grelina desacetilada, que também foi observado neste grupo, já que os neurônios NPY apresentam um importante papel nas

funções orexigênicas da grelina (THOMPSON ET AL., 2003). Além disso, o aumento nos níveis de NPY hipotalâmico após uma semana de estresse pode estar associado ao aumento da liberação de glicocorticoides, que podem agir no SNC modulando a ingestão alimentar, possivelmente via ativação do NPY (DALLMAN ET AL., 1993; BHATNAGAR ET AL., 2000; DALLMAN ET AL., 2005). É possível que esse aumento induzido pelo estresse no período pré-púbere sobre os níveis plasmáticos de grelina e de NPY hipotalâmico em ratos machos jovens possa estar relacionado com o aumento do consumo calórico observado durante este período. Com relação às fêmeas, não houve alterações nos níveis desses peptídeos, mostrando mais uma vez diferenças sexo-expecíficas.

Outro hormônio que apresentou uma diferença com relação ao sexo foi a adiponectina. Este hormônio, que é uma adipocina produzida pelo tecido adiposo, tem por função atuar no metabolismo de lipídeos e da glicose, controlando a homeostasia energética e participando da regulação endócrina (KORNER ET AL., 2007; MICHALAKIS ET AL., 2012). Além disso, apresenta propriedades anti-diabética, anti-aterogênica e anti-inflamatória (MATSUZAWA ET AL., 2010; SHIBATA ET AL., 2012), estando essas propriedades possivelmente relacionadas com os efeitos benéficos desse hormônio sobre o sistema cardiovascular (HUI ET AL., 2012). Neste estudo, foi observado um aumento nos níveis plasmáticos da adiponectina, induzido pela exposição ao estresse durante o período pré-púbere em ratas fêmeas jovens, enquanto nos machos jovens houve uma diminuição causada pelo estresse nos níveis deste hormônio. Este aumento nos níveis plasmáticos de adiponectina encontrado apenas nas fêmeas poderia estar relacionado ao fato de que elas parecem ser mais protegidas ou se adaptam melhor aos efeitos do estresse. Quando esse mesmo parâmetro foi avaliado na idade adulta, não houve diferenças significativas com relação ao estresse. No entanto, a exposição

precoce a uma dieta rica em carboidratos simples levou a uma redução nos níveis plasmáticos de adiponectina na idade adulta apenas nos ratos machos. A hipoadiponectinemia está intimamente associada com diabetes do tipo 2, desequilíbrios nos lipídeos, hipertensão e também algumas doenças inflamatórias (MICHALAKIS ET AL., 2012). Esses níveis plasmáticos reduzidos de adiponectina podem ajudar a explicar alguns resultados encontrados neste estudo, incluindo aumento do peso corporal, aumento do depósito de gordura visceral e da glicemia, os quais, juntamente com a redução da adiponectina, podem ser fatores de risco para síndrome metabólica (KYROU ET AL., 2006). Embora neste estudo não fossem significativamente diferentes todos os parâmetros que pudessem indicar síndrome metabólica nestes animais, as alterações encontradas podem indicar maior susceptibilidade a desenvolver esta condição.

Além de apresentar efeitos periféricos, a adiponectina também contribui para a regulação central da ingestão alimentar e da homeostasia energética (KUBOTA ET AL., 2007), e pode ter outras funções no encéfalo. A expressão dos receptores de adiponectina foi encontrada em neurônios hipocampais de camundongos, na linhagem celular GT1-7, derivada de neurônios hipotalâmicos de ratos, e na hipófise, nos núcleos da base e no hipotálamo de humanos (JEON ET AL., 2009; GUILLOD-MAXIMIN ET AL., 2009; PSILOPANAGIOTI ET AL., 2009; WEN ET AL., 2008). Camundongos nocautes para a adiponectina mostraram maior prejuízo neurológico após a reperfusão isquêmica, sugerindo que a adiponectina apresenta uma atividade neuroprotetora (NISHIMURA ET AL., 2008). Outros estudos também mostraram que a adiponectina está intimamente relacionada à proteção contra a produção de espécies reativas do oxigênio e também contra apoptose no hipocampo, sugerindo um importante papel de

neuroproteção dos neurônios hipocampais em condições neurodegenerativas aguda e crônica (QIU ET AL., 2011; WAN ET AL., 2009).

Além da possível relação entre a redução na adiponectina e a indução de neurodegeneração e excitotoxicidade, mostradas nos estudos citados acima, cabe evidenciar que a exposição exacerbada ao estresse também pode desencadear neurodegeneração no SNC. A exposição a eventos estressantes leva a um aumento na liberação de glicocorticoides devido à ativação do eixo HHA. Por sua vez, os glicocorticoides participam do controle da atividade deste eixo através de um mecanismo de retroalimentação negativa, o qual permite que esses hormônios altamente lipossolúveis liguem-se aos seus receptores, presentes em distintas estruturas encefálicas, tais como córtex pré-frontal e hipocampo, além de outras. Elevados níveis de glicocorticoides liberados em resposta ao estresse podem levar a um aumento na geração de espécies reativas do oxigênio, que pode induzir disfunção mitocondrial, prejuízo na bioenergética, apoptose, danos a precursores neuronais e prejuízo na neurogênese (KROEMER ET AL., 1997; MITRA ET AL., 2005; PAPADOPOULOS ET AL., 1997).

Devido à diminuição nos níveis plasmáticos de adiponectina, observada apenas em ratos machos adultos, e a sua possível influência sobre o metabolismo celular, assim como a possível influência do estresse sobre esse metabolismo, tornou-se fundamental estudarmos como estaria o a função mitocondrial e o equilíbrio oxidativo em estruturas encefálicas fundamentais (córtex pré-frontal e hipocampo) para o controle da resposta ao estresse em ratos machos ao longo do desenvolvimento. Outro aspecto importante e relevante para a escolha destas duas estruturas cerebrais para nosso estudo é que a maturação e a plasticidade durante o desenvolvimento são altamente variáveis em

diferentes regiões cerebrais, incluindo aquelas envolvidas no processamento e na regulação do estresse e da emoção: o córtex pré-frontal, por exemplo, possui uma maturação tardia (GOGTAY ET AL., 2004), e o hipocampo apresenta um aumento na neurogênese e densidade dos espinhos dendríticos antes da puberdade, o que diminui na idade adulta (HE e CREWS, 2007; YILDIRIM ET AL., 2008). Por isso, intervenções precoces podem alterar, nessas estruturas, a programação do metabolismo celular durante o desenvolvimento até a idade adulta.

No capítulo II da presente tese, foram avaliados parâmetros do metabolismo celular no córtex pré-frontal de ratos machos de diferentes idades (juvenis e adultos). De uma forma geral, foram observadas alterações na atividade das enzimas antioxidantes, na atividade do complexo IV da cadeia transportadora de elétrons e na atividade da enzima Na^+,K^+-ATPase nos ratos submetidos ao estresse por isolamento social no período pré-púbere. No entanto, esses efeitos não foram prevenidos pelo consumo de uma dieta palatável, mostrando que a redução da resposta ao estresse que tem sido sugerido estar relacionada ao consumo de alimentos altamente palatáveis não parece atuar ou não é capaz de atenuar esse tipo de efeito. Com relação aos resultados observados nos ratos machos jovens, o estresse por isolamento social aumentou a atividade da SOD, a atividade do complexo IV da cadeia transportadora de elétrons, e a razão SOD/GPx. Já a dieta palatável aumentou a atividade da enzima superóxido dismutase e da Na^+,K^+-ATPase . Além desses efeitos isolados, quando os dois fatores (estresse e dieta) estavam juntos houve um aumento na atividade da enzima catalase. Nos animais adultos, o estresse diminuiu as atividades das enzimas Na^+,K^+-ATPase, catalase e aumentou a atividade da enzima SOD e do complexo IV da cadeia transportadora de elétrons, assim como as razões SOD/GPx e SOD/CAT.

Numerosos processos fisiológicos e patológicos, tais como estresse psicológico ou emocional, transtornos psiquiátricos, envelhecimento, excessos de ingestão calórica, infecções, entre outros, aumentam a concentração de substâncias oxidantes, conhecidas como radicais livres ou espécies reativas (TSALUCHIDU ET AL., 2008). Além disso, não só pode ocorrer um aumento dessas substâncias como também um desequilíbrio dos sistemas antioxidantes, como é observado em alguns estudos em que os animais são submetidos cronicamente a um estressor psicológico (KROLOW ET AL., 2010; NOSCHANG ET AL., 2009). No capítulo II desta tese, observamos que ratos jovens submetidos ao isolamento social e à dieta palatável apresentaram aumento da atividade da SOD. Houve uma interação entre estresse e dieta palatável aumentando a atividade da CAT neste mesmo período do desenvolvimento. No entanto, o estresse aplicado no período pré-púbere induziu uma diminuição na atividade da CAT quando os animais atingiram a idade adulta. Com relação às razões SOD/GPx e SOD/CAT, o estresse por isolamento social induziu aumento nessas razões quando os animais eram jovens, e este aumento permaneceu até a idade adulta. Já com relação à razão SOD/CAT, ela foi aumentada pelo estresse apenas na idade adulta. Os resultados abordados acima sugerem um desequilíbrio no sistema enzimático antioxidante no córtex pré-frontal. Um aumento a longo-prazo na atividade da SOD sem qualquer alteração na atividade da GPx ou da CAT sugere possivelmente um aumento na produção de H_2O_2. Sabe-se que esta espécie reativa pode apresentar um papel fisiológico de sinalização nos processos celulares, como também pode facilitar a produção de $OH^{\cdot}$, uma molécula altamente oxidante que pode danificar o ADN celular.

Uma vez que o desequilíbrio oxidativo pode estar intimamente relacionado com disfunção mitocondrial, foi avaliada a atividade dos complexos da cadeia transportadora

de elétrons. O estresse por isolamento social aumentou a atividade do complexo citocromo oxidase (IV) nos juvenis, e esse aumento permaneceu na idade adulta. Os glicocorticoides (GCs), os quais são liberados durante a resposta ao estresse, podem direta ou indiretamente afetar as funções mitocondriais. Além disso, foi demonstrado que a energia celular pode ser regulada pelos GCs via receptores de glicocorticoides, que regulam ambos os genes nucleares e mitocondriais envolvidos na biossíntese das enzimas da cadeia transportadora de elétrons (TSIRIYOTIS ET AL., 1997). Um estudo recente mostrou que o estresse aumentou, no córtex pré-frontal, a expressão dos genes de citocromo oxidase 1 e 3, subunidades catalíticas da citocromo oxidase, enzima envolvida no transporte mitocondrial de elétrons (ADZIC ET AL., 2009). Portanto, é possível que o efeito encontrado nesta tese de aumento na atividade do complexo IV possa ser atribuído à expressão da citocromo oxidase, induzida pelos GCs liberados em resposta ao estresse por isolamento social. Também se pode especular que este possível aumento na expressão da citocromo oxidase, com consequente aumento em sua atividade, pode ter induzido uma maior produção de ânion superóxido na mitocôndria, levando a um aumento da SOD, conforme observado nos animais estressados. No entanto, não houve alteração na massa ou no potencial mitocondrial nesta estrutura.

Outro achado importante neste capítulo refere-se à atividade da atividade enzima Na^+,K^+-ATPase. A dieta palatável aumentou a atividade desta enzima apenas nos ratos machos jovens; quando essa dieta foi retirada, permanecendo apenas o alimento padrão até a idade adulta, nenhuma alteração foi encontrada na atividade desta enzima. De modo contrário ao resultado observado neste trabalho de tese, um estudo prévios mostrou que a exposição a alimentos palatáveis na idade adulta reduz a atividade da Na^+,K^+-ATPase (da S BENETTI ET AL., 2010). Uma explicação para essa

discrepância poderia ser o fato de que, no presente trabalho, no período em que os animais foram expostos a esse alimento palatável, estruturas cerebrais, como por exemplo o córtex pré-frontal, estão em desenvolvimento, e alterações na densidade sináptica estão ocorrendo, de modo que uma intervenção durante este período poderia apresentar uma resposta diferenciada sobre a atividade desta enzima-chave para o metabolismo neural. Diferentemente da dieta palatável, o estresse por isolamento social diminuiu a atividade da Na^+,K^+-ATPase apenas na idade adulta dos ratos. Alguns estudos já demonstraram que o estresse diminui a atividade desta enzima (CREMA ET AL., 2010; de VASCONCELLOS ET AL., 2005), um efeito que é revertido quando o estresse é interrompido (de VASCONCELLOS ET AL., 2005). Esta redução na atividade da Na^+,K^+-ATPase pode comprometer a neurotransmissão, uma vez que essa enzima é responsável pela geração do potencial de membrana necessário para manutenção da excitabilidade neural. Não se pode atribuir a inibição da atividade dessa enzima à redução da produção de energia, pois o estresse aumentou a atividade da enzima citocromo oxidase e não houve outros efeitos sobre os complexos da cadeia transportadora de elétrons. Esta diminuição da atividade da enzima Na^+,K^+-ATPase pode estar relacionada com o estado oxidativo alterado, pois neste estudo foi observado um desequilíbrio oxidativo. Essa hipótese é válida, pois alguns estudos mostraram que a enzima em questão é susceptível ao ataque por radicais livres (DOBROTA ET AL., 1999), e também à redução da atividade de enzimas antioxidantes (STRECK ET AL., 2001).

Nos capítulos III e IV desta tese, foram avaliados parâmetros do metabolismo celular no hipocampo de ratos machos ao longo do desenvolvimento. Alguns parâmetros avaliados apresentaram alterações similares àquelas previamente observadas

no córtex, como, por exemplo, o desequilíbrio entre os sistemas antioxidantes, que foi evidente tanto no córtex pré-frontal quanto no hipocampo a longo-prazo. Por outro lado, de modo distinto dos resultados observados para o córtex pré-frontal com relação à função mitocondrial, o hipocampo de ratos submetidos ao estresse por isolamento social e à dieta palatável apresentou alterações mais expressivas nesta organela, sugerindo que as estruturas encefálicas estudadas apresentam vulnerabilidade mitocondrial distinta no que se refere aos efeitos da exposição a fatores ambientais precoces, tais como a exposição ao isolamento social e à dieta palatável. Para uma sinopse dos resultados encontrados em estruturas cerebrais, ver as tabelas 1 e 2 no Anexo 1 desta tese. Pode-se observar que não há discrepâncias marcantes entre os efeitos dos fatores estudados sobre as duas estruturas, no entanto, como comentado acima, o hipocampo mostrou-se mais susceptível ao estresse oxidativo e, especialmente, à disfunção mitocondrial.

Vários estudos têm demonstrado que o estresse resulta no desequilíbrio dos sistemas antioxidantes enzimáticos, podendo levar ao estresse oxidativo (KROLOW ET AL., 2010; McINTOSH e SAPOLSKY, 1996; NOSCHANG ET AL., 2009). No hipocampo de ratos jovens, a exposição ao estresse aumentou a produção de radicais livres. Houve uma interação entre estresse e dieta palatável, aumentando a atividade da SOD e, consequentemente, levando a um aumento na razão SOD/GPx. Na tentativa de verificar se os efeitos do estresse e da dieta palatável eram mantidos a longo-prazo, quando os animais não mais eram estressados e a dieta passava a uma dieta padrão, foram avaliados os mesmos parâmetros de estresse oxidativo no hipocampo de ratos machos adultos. Alguns parâmetros permaneceram alterados, como por exemplo, o aumento na produção de radicais livres os quais foram induzidos pela exposição precoce ao estresse. Além disso, mesmo na idade adulta, continua a haver uma interação

entre estresse e dieta, aumentando a atividade da enzima SOD; também observamos que o estresse aumentou as razões SOD/GPx e SOD/CAT e a dieta palatável aumentou a razão SOD/GPx. Esses achados sugerem que o estresse no período pré-púbere induziu desequilíbrio oxidativo a longo-prazo, principalmente por aumentar a produção de radicais livres, induzir um aumento na atividade da SOD, efeito que é exacerbado quando associado à dieta palatável. Com já foi discutido no capítulo II desta tese, um aumento na produção de H_2O_2 sem qualquer alteração nas atividades das enzimas GPx e CAT pode levar ao aumento do radical hidroxila, o qual, por sua vez, pode danificar o ADN celular. Nota-se que esses achados do estresse induzindo a longo-prazo um desequilíbrio oxidativo foram também observados no córtex pré-frontal, mostrando que possivelmente a exposição precoce a esse fator ambiental pode programar o estado oxidativo a longo-prazo no SNC. Curiosamente, a dieta palatável quando oferecida precocemente, pareceu promover uma ativação dos sistemas antioxidantes, havendo aumento do conteúdo de tióis e da atividade da GPx nos juvenis. No entanto, quando a oferta dessa dieta é retirada e apenas o alimento padrão é oferecido aos animais, ocorre uma aumento na razão SOD/GPx na idade adulta, mostrando que possivelmente a dieta palatável oferecida no início da vida pode levar a consequências que incluem o aumento a longo-prazo no estado oxidativo.

Devido ao desequilíbrio oxidativo observado no hipocampo de ratos jovens e adultos tornou-se digno de estudo avaliar o índice de quebras ao ADN celular no hipocampo destes animais. As quebras ao ADN celular podem ser induzidas por nucleases ativadas por cálcio e/ou por espécies reativas do oxigênio, principalmente pelo radical $OH^{\bullet}$ formado pela reação de H_2O_2 com íons metálicos, ligados ao ADN celular (DARLEY-USMAR e HALLIWELL, 1996). Nos ratos jovens, o estresse

induziu um aumento no índice de quebras ao ADN celular, possivelmente devido ao desequilíbrio oxidativo. Já na idade adulta destes animais, foi visto um aumento marcante no índice de quebras ao ADN celular induzido pela exposição precoce a uma dieta palatável. Alguns estudos têm apresentado evidências de que o excesso de ingestão de alimentos palatáveis pode levar à fragmentação do ADN celular (KROLOW ET AL., 2010; OLIVO-MARSTON ET AL., 2008). Curiosamente esse efeito não foi observado quando os animais eram jovens, mas apenas quando este alimento foi retirado e substituído pelo alimento padrão. No capítulo1 foi observado que os ratos machos que haviam recebido alimento palatável no início da vida seguiram consumindo mais alimento padrão até a idade adulta. Além disso, apresentaram uma diminuição na adiponectina plasmática, que pode estar relacionada com uma maior susceptibilidade a danos oxidativos, os quais, como discutido acima, poderiam estar relacionados ao aumento no dano ao ADN. Mais estudos seriam necessários para verificar essa possível correlação. No entanto, quando os dois fatores, estresse e dieta palatável, estavam juntos, houve uma redução marcante no índice de quebras ao ADN celular no hipocampo de ratos adultos. Essa interação poderia estar relacionada ao efeito de redução do estresse que a ingestão de alimentos confortantes, ricos em carboidratos simples, pode apresentar em ratos estressados cronicamente (FACHIN ET AL., 2008; PECORARO ET AL., 2004).

Outro ponto importante para esse estudo foi verificar as possíveis alterações que o estresse por isolamento social e a oferta de uma dieta palatável no período pré-púbere induziram sobre a mitocôndria. Sabe-se que a mitocôndria tem um papel vital na célula e que é uma das primeiras organelas a responder ao estresse, e essa resposta envolve alterações importantes nas funções mitocondriais, na capacidade de ajuste da

bioenergética, termogênese e respostas oxidativas e/ou apoptóticas **(**MANOLI ET AL., 2007**).** A resposta mitocondrial a um evento estressante pode ser bifásica: uma exposição de curto-prazo induz alterações nos níveis de glicocorticóides associadas com a indução da biogênese mitocondrial e da atividade enzimática de seletas subunidades dos complexos da cadeia respiratória, enquanto que, se a exposição for mais prolongada, os glicocorticóides podem causar disfunção na cadeia respiratória, aumento na geração de EROs, anormalidades na estrutura mitocondrial, apoptose e morte celular (ALESCI ET AL., 2006; DUCLOS ET AL., 2004; MANOLI ET AL., 2005; ORZECHOWSKI ET AL., 2002). Nas células hipocampais de ratos jovens, o estresse induziu um aumento no número de células com alto potencial e houve uma diminuição no número de células com baixa massa mitocondrial, sugerindo uma possível biogênese mitocondrial; no entanto, não houve alteração na atividade dos complexos respiratórios. Por outro lado, esses achados também podem sugerir que o aumento do potencial mitocondrial esteja associado com desequilíbrio oxidativo: um aumento nesse potencial está associado com aumento de vazamento de elétrons na cadeia respiratória, levando a aumento de emissão de ERO e diminuição da fosforilação ativa (ADREYEV ET AL., 2005), o que foi de fato observado no capítulo III desta tese. Sendo assim, o aumento do potencial mitocondrial poderia ajudar a explicar o desequilíbrio oxidativo observado nos animais estressados.

Com relação ao efeito do acesso a uma dieta palatável sobre o potencial mitocondrial, é interessante que essa dieta aumentou o número de células com o potencial alto na mitocôndria e a atividade dos complexos de I-III da cadeia transportadora de elétrons. É possível que este aumento seja devido a uma resposta adaptativa, pois mais glicose provinda dessa dieta rica em carboidratos simples está

sendo oxidada e, com isso, ocorre um aumento nos doadores de elétrons para a cadeia respiratória e, consequentemente, um aumento no potencial de membrana mitocondrial.

Já nos animais adultos, a resposta foi diferente, havendo interação entre estresse e dieta palatável, de modo que, quando esses dois fatores estavam associados, havia uma diminuição da massa e do potencial mitocondrial, ou seja, diminuiu a biogênese de mitocôndrias. Como discutimos anteriormente, ambos os fatores (estresse e dieta), quando juntos, potencializam a atividade da SOD. Esses resultados, o aumento da atividade da SOD e a diminuição da massa e do potencial de membrana mitocondrial, podem estar associados. Não é possível, no entanto, determinar com segurança se os efeitos induzidos pela exposição ao estresse juntamente com dieta palatável são adaptativos ou mal-adaptativos com relação à mitocôndria. É interessante observar que apenas houve alterações na massa e no potencial mitocondrial das células hipocampais, e não houve alterações com relação a esses parâmetros nas células do córtex pré-frontal, sugerindo distinta vulnerabilidade entre as estruturas encefálicas estudadas.

Interações entre estresse e dieta também foram observadas no hipocampo dos ratos adultos com relação às atividades dos complexos II e IV da cadeia transportadora de elétrons. Quando ambos os fatores estavam juntos, as atividades desses complexos retornaram aos níveis do grupo controle. Nesses achados, mais uma vez, observamos que a exposição à dieta palatável atenuou os efeitos do estresse. É interessante considerar que o estresse por isolamento social mostrou uma tendência em aumentar a atividade do complexo IV nas células hipocampais, assim como foi visto no córtex pré-frontal. Sendo assim, podemos especular que a exposição aos glicocorticoides pode estar aumentando a síntese da citocromo oxidase na mitocôndria, como sugerido por Adizic et al. (2009). No entanto, esse efeito parece ser mais marcante no córtex pré-

frontal do que no hipocampo, pois essa alteração foi percebida no córtex deste o período pré-púbere até a idade adulta.

Alterações na fisiologia da mitocôndria e dano celular oxidativo induzido pelo estresse são considerados eventos centrais da apoptose (FRANKLIN ET AL., 2011; NIIZUMA ET AL., 2010; VALKO ET AL., 2006). Com base nisso, avaliamos, nas células do hipocampo de ratos jovens e adultos submetidos a estresse por isolamento social e a uma dieta palatável, a possível susceptibilidade à morte celular induzida por esses fatores ambientais durante o desenvolvimento. O isolamento social no período pré-púbere induziu um aumento no número de células em apoptose inicial, enquanto que, na idade adulta, levou a um aumento no número de células necróticas. Durante a morte celular, evidências indicam um aumento na função mitocondrial (KLUZA ET AL., 2004; MAHYAR-ROEMER ET AL., 2001), embora outros estudos tenham mostrado uma ligação entre a diminuição no potencial mitocondrial e apoptose (GOTTEIB ET AL., 2000; SCARLETT ET AL., 2000). É possível que este parâmetro seja dependente do estágio de morte celular apoptótica. Durante o período pré-púbere, intensas modificações ocorrem no SNC (MCCORMICK e MATHEWS, 2007) e um aumento de células em apoptose inicial pode sugerir algum tipo de reorganização dos circuitos, induzida pelo estresse. É interessante observar que a interação com a dieta palatável levou a uma redução da apoptose, mostrando novamente que o acesso a tal dieta parece reduzir os efeitos do estresse, e poderíamos especular que tal reorganização de circuitos não seria tão necessária. Contrapondo esses achados, na idade adulta foi observado um aumento no número de células em necrose, mostrando que, a longo prazo, a via envolvida não é mais a apoptose e sim a necrose, que pode estar associada à diminuição no número de mitocôndrias, como foi observado anteriormente. Da mesma

maneira, a interação com a dieta palatável mostra o retorno aos níveis do controle, mostrando que essa dieta pode reduzir a resposta ao estresse (ver figura 1 do anexo).

Já com relação ao efeito do alimento palatável *per se*, houve alterações marcantes na idade adulta sobre os parâmetros de morte celular. A dieta palatável diminuiu o número de células vivas e aumentou a apoptose inicial, novamente mostrando que a exposição precoce a esse alimento rico em carboidratos simples parece ter programado a longo-prazo o início da morte celular.

É interessante observar que, no que tange aos efeitos da dieta palatável sobre vários dos parâmetros avaliados nos jovens e nos adultos, observamos que, enquanto a dieta parece não apresentar efeitos marcantemente desfavoráveis nos juvenis, o acesso naquele período a uma dieta palatável apresenta, *per se*, na idade adulta, alguns efeitos deletérios, aumentando o índice de quebras do ADN, diminuindo o número de células vivas e aumentando a apoptose. Uma questão que poderia ser levantada diz respeito ao fato de que a dieta foi interrompida. A retirada de uma dieta palatável poderia funcionar, neste caso, como um fator de frustração para os animais? Essa frustração poderia ser em parte responsável pelos efeitos a longo-prazo da dieta? Essas questões são relevantes e necessitariam de estudos adicionais para serem respondidas. Poderíamos considerar que tal fenômeno (a frustração) provavelmente se apresentaria de modo diferente em animais estressados que recebiam dieta palatável ou animais que apenas tinham acesso a tal dieta, sem estarem estressados.

Devido a todas as alterações encontradas no metabolismo celular hipocampal, causadas pelo estresse e/ou pela dieta palatável, foi relevante avaliar se essas intervenções precoces poderiam prejudicar a memória espacial destes animais. Estudos mostraram que os estressores durante fases iniciais do desenvolvimento podem afetar o

desempenho cognitivo em ratos (HODES e SHORS, 2005; TSOORY e RICHTER-LEVIN, 2005). Além disso, sabe-se que o hipocampo é uma estrutura cerebral importante para o aprendizado e a memória, e sua fisiologia e morfologia são sensíveis aos efeitos de estressores agudos e crônicos (TSOORY e RICHTER-LEVIN, 2005; HOWLAND e WANG, 2008). Observamos que, durante as sessões de aprendizado na tarefa do labirinto aquático de Morris, os animais que foram isolados e recebiam alimento palatável aumentaram a latência para encontrar a plataforma. No entanto, no dia do teste, não observamos efeitos sobre a memória, apenas uma redução induzida por ambos os fatores na velocidade do nado. Frente a esse resultado, foi avaliada a atividade motora, e os resultados sugerem que o efeito na latência para encontrar a plataforma durante a fase do aprendizado no labirinto aquático não pode ser atribuído ao prejuízo cognitivo e nem mesmo motor, pois nem a memória, nem a atividade motora foram alteradas. No entanto, os efeitos observados podem estar relacionados com outros fatores, tais como emoção ou motivação, sendo necessários mais estudos para confirmar essa hipótese.

Na tentativa de compreender o mecanismo pelo qual a dieta palatável pode prevenir alguns efeitos induzidos pelo estresse sobre o metabolismo celular, foi avaliado o imunoconteúdo dos receptores de glicocorticoides no hipocampo de ratos machos adultos. Esses receptores estão presentes em grande número no hipocampo, participando da retroalimentação negativa do eixo HHA. Neste estudo, observamos um aumento induzido pela exposição precoce a uma dieta palatável sobre o conteúdo desses receptores. Podemos especular que o acesso a uma dieta palatável na pré-puberdade programa a resposta do eixo HHA, de modo que os níveis dos glicocorticoides retornem mais rapidamente para valores normais após a exposição ao estresse, reduzindo, assim,

as consequências de tal estressor. Esses resultados ajudariam a explicar várias das interações encontradas neste estudo.

Os achados da presente tese contribuíram para mostrar que intervenções precoces no desenvolvimento, tais como a exposição ao isolamento social e a uma dieta palatável, podem, de certa forma, programar tanto o metabolismo periférico quanto o metabolismo celular neural. Com relação ao metabolismo periférico, a exposição à dieta palatável empregada neste trabalho parece ter causado efeitos mais marcantes a longo-prazo, principalmente nos ratos machos. No entanto, com relação aos achados referentes ao metabolismo celular neural, foi verificado que as exposições precoces a fatores ambientais programaram a longo-prazo alterações no metabolismo celular em diferentes aspectos com relação às estruturas encefálicas estudadas. Essa programação está provavelmente relacionada a modificações epigenéticas, levando a expressão distinta de certos genes nos animais que passaram por tais experiências precoces. Assim, a compreensão desses parâmetros alterados no metabolismo torna-se relevante para que futuramente possamos tentar reprogramar tais alterações. Um melhor entendimento desses processos pode levar ao desenvolvimento de estratégias para evitar o aparecimento de patologias na vida adulta, associadas a interferências no período pré-púbere.

CONCLUSÕES

- ✓ O isolamento social e a dieta palatável são fatores ambientais que, aplicados no período pré-púbere em ratos machos e fêmeas, causam uma resposta no metabolismo periférico de maneira sexo-específica. As fêmeas, mesmo antes da puberdade, foram mais propensas a utilizar alimentos confortantes quando expostas ao estresse. Já ratos machos foram mais propensos à programação metabólica a longo-prazo, induzida pela exposição precoce ao alimento palatável, levando a alterações que sugerem maior susceptibilidade a um quadro de síndrome metabólica, situação que pode estar relacionada à hipoadiponectinemia.
- ✓ O isolamento social programou o metabolismo celular a longo-prazo no córtex pré-frontal de ratos machos. Aumentou o desequilíbrio entre os sistemas antioxidantes e induziu uma redução da enzima Na, K^+-ATPase. A exposição precoce à dieta palatável não foi capaz de prevenir esses efeitos.
- ✓ O isolamento social durante o período pré-púbere induziu, nas células do hipocampo de ratos jovens, alterações no metabolismo celular, com desequilíbrio oxidativo e aumento do potencial mitocondrial, do índice de quebras ao ADN e da apoptose. O consumo da dieta palatável aumentou atividade de enzimas antioxidantes e preveniu diversos dos efeitos do estresse, indicando que a oferta de dieta palatável durante a exposição ao estresse por isolamento social pode funcionar com um mecanismo compensatório ou pode facilitar a adaptação ao estresse durante fases iniciais da vida.
- ✓ O isolamento social no período pré-púbere induziu desequilíbrio oxidativo e aumentou o número de células necróticas no hipocampo de ratos adultos. A ingestão de alimento palatável durante a exposição ao estresse atenuou alguns efeitos do estresse, incluindo as alterações nas atividades dos complexos

respiratórios, no índice de fragmentação do ADN celular e no número de células necróticas. Esses efeitos podem estar relacionados com o aumento no imunoconteúdo de receptores de glicocorticoides no hipocampo, induzido pela dieta palatável. No entanto, ambos os fatores ambientais, estresse e dieta, podem induzir, *per se*, alterações mal-adaptativas quando aplicados isoladamente.

Em suma, esta tese mostrou que intervenções precoces durante o desenvolvimento do animal podem programar o metabolismo ao longo da vida. Os machos foram mais susceptíveis ao consumo de alimento palatável quando avaliados parâmetros do metabolismo a longo-prazo. Das estruturas cerebrais avaliadas, o hipocampo mostrou maior susceptibilidade. Houve claros efeitos sobre o equilíbrio oxidativo e a função mitocondrial mesmo na idade adulta, muito tempo após as intervenções terem cessado, e a dieta palatável foi capaz de prevenir vários dos efeitos do estresse no período pré-púbere.

PERSPECTIVAS

- ✓ Avaliar parâmetros de viabilidade celular e índice de fragmentação do ADN quando uma dieta palatável é oferecida cronicamente desde a pré-puberdade até a idade adulta;

- ✓ Avaliar a relevância da adiponectina no desencadeamento dos efeitos observados por meio de estudos farmacológicos e bioquímicos, verificando a possível prevenção de alterações relacionadas ao metabolismo periférico e neural;

- ✓ No modelo de estresse no período pré-púbere empregado nesta tese, com e sem acesso a uma dieta palatável, investigar comportamentos relacionados à motivação na idade adulta (especialmente comportamento alimentar) e vias de sinalização relacionadas à motivação;

- ✓ Investigar a influência de hormônios gonadais sobre parâmetros metabólicos periféricos e sobre o metabolismo neural em estruturas cerebrais relacionadas ao estresse em animais submetidos ao isolamento no período pré-púbere com e sem acesso a uma dieta palatável;

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ANEXO

Tabela 1: Efeitos sobre o metabolismo celular neural, induzidos pela exposição precoce ao isolamento social e a uma dieta palatável encontrados em diferentes estruturas encefálicas de ratos machos jovens.

Parâmetros Avaliados	Hipocampo	Córtex Pré-Frontal
SOD	Interação ↑	ESTRESSE ↑ DIETA ↑
CAT	----------	Interação ↑
GPx	DIETA ↑	----------
DCF	ESTRESSE ↑	----------
Tióis	ESTRESSE ↑ DIETA ↑	----------
SOD/GPx	ESTRESSE ↑	ESTRESSE ↑
SOD/CAT	----------	----------
COMETA	ESTRESSE ↑	Não avaliado
MASSA BAIXA	ESTRESSE↓	----------
MASSA ALTA	----------	----------
POTENCIAL BAIXO	ESTRESSE↓	----------
POTENCIAL ALTO	ESTRESSE ↑ DIETA ↑	----------
COMPLEXO II	----------	----------
COMPLEXO I-III	DIETA ↑	----------
COMPLEXO IV	----------	ESTRESSE ↑
Na^+,K^+-ATPASE	Não avaliado	DIETA ↑
CÉLULAS VIVAS	Interação-retorno aos níveis do controle	Não avaliado
APOPTOSE INICIAL	ESTRESSE ↑ Interação↓	Não avaliado
APOPTOSE FINAL	----------	Não avaliado
NECROSE	ESTRESSE↓ Interação↓	Não avaliado

Tabela 2: Efeitos sobre o metabolismo celular neural, induzidos pela exposição precoce ao isolamento social e a uma dieta palatável encontrados em diferentes estruturas encefálicas de ratos machos <u>adultos</u>.

Parâmetros Avaliados	Hipocampo	Córtex Pré-Frontal
SOD	Interação ↑	ESTRESSE ↑
CAT	----------	ESTRESSE↓
GPx	----------	----------
DCF	ESTRESSE ↑	----------
Tióis	----------	----------
SOD/GPx	ESTRESSE ↑ DIETA ↑	ESTRESSE ↑
SOD/CAT	ESTRESSE ↑	ESTRESSE ↑
COMETA	DIETA ↑ Interação ↓	Não avaliado
MASSA BAIXA	Interação ↑	----------
MASSA ALTA	Interação ↓	----------
POTENCIAL BAIXO	----------	----------
POTENCIAL ALTO	Interação ↓	----------
COMPLEXO II	Interação-retorno aos níveis do controle	----------
COMPLEXO I-III	----------	----------
COMPLEXO IV	Interação-retorno aos níveis do controle	ESTRESSE ↑
Na^+,K^+-ATPASE	Não avaliado	ESTRESSE ↓
CÉLULAS VIVAS	DIETA ↓	Não avaliado
APOPTOSE INICIAL	DIETA ↑	Não avaliado
APOPTOSE FINAL	ESTRESSE ↓	Não avaliado
NECROSE	ESTRESSE ↑ Interação-retorno aos níveis do controle	Não avaliado
RECEPTORES DE GLICOCORTICOIDES	DIETA ↑	Não avaliado

Figura 1: Comparativo das alterações no metabolismo celular ao longo do desenvolvimento no hipocampo de ratos machos.