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INFLUÊNCIA DA SUPLEMENTAÇÃO DE ÁCIDOS GRAXOS EM DIFERENTES FASES DE DESENVOLVIMENTO DE RATOS: AVALIAÇÕES COMPORTAMENTAIS, BIOQUÍMICAS E MOLECULARES

Santa Maria, RS
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Tese apresentada ao Curso de Pós-Graduação em Farmacologia, Área de Concentração em Neuropsicofarmacologia, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Doutor em Farmacologia.**

Orientadora: Prof[a]. Dr[a]. Marilise Escobar Bürger

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Aprovado em 01 de março de 2016:

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2016

Esta tese é dedicada à minha família.

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“Que seu remédio seja seu alimento,

e que seu alimento seja seu remédio”

Hipócrates

RESUMO

INFLUÊNCIA DA SUPLEMENTAÇÃO DE ÁCIDOS GRAXOS EM DIFERENTES FASES DE DESENVOLVIMENTO DE RATOS: AVALIAÇÕES COMPORTAMENTAIS, BIOQUÍMICAS E MOLECULARES

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Os ácidos graxos (AG) são constituintes importantes dos fosfolipídeos das membranas e desempenham importantes funções no sistema nervoso central (SNC). Evidências sugerem que a nutrição materna durante períodos iniciais de desenvolvimento está diretamente relacionada ao adequado desenvolvimento do feto, recém-nascido e adulto uma vez que neste período ocorre a transferência dos ácidos graxos poli-insaturadosde cadeia longa através da placenta e do leite, enquanto que uma nutrição materna inadequada pode alterar ambos os parâmetros morfológicos e fisiológicos dos filhotes. Nas décadas recentes, foram observadas mudanças nos hábitos alimentares, as quais possibilitaram o aumento do consumo de ácidos graxos *trans*, em detrimento do consumo de ácidos graxos essenciais, principalmente os da família n-3. Tais alterações podem prejudicar a neuroplasticidade e favorecer o desenvolvimento de doenças neuropsiquiátricas. Além disso, situações frequentes de estresse devido a pressão do mundo exterior também podem estar associadas com o desenvolvimento de doenças que envolvem o SNC. Neste estudo, nós avaliamos a influência do consumo ou suplementação de diferentes ácidos graxos durante o período perinatal sobre alterações comportamentais, bioquímicas e moleculares em ratos adultos. A partir dos resultados apresentados no artigo 1, foi possível observar os efeitos benéficos do consumo de uma dieta enriquecida com azeite de oliva (20%) em diferentes fases do desenvolvimento, como gestação, lactação ou após o desmame dos filhotes até a idade adulta. No presente estudo, contatou-se a redução do peso corporal e estresse oxidativo em todos os períodos analisados, além do aumento da expressão de fatores neurotróficos como BDNF e FGF-2 após consumo da dieta contendo azeite de oliva durante o período perinatal. No artigo 2, foi avaliada a relação entre o consumo prolongado de gordura vegetal hidrogenada (GVH), rica em AG *trans,* em diferentes períodos da vida de ratos, e o desenvolvimento de sintomas de hiperatividade. O consumo de gordura *trans,* por 10 meses, bem como a suplementação durante a gravidez e lactação ao longo de duas gerações sequenciais de animais induziu comportamento ativo no teste do nado forçado. Também, a suplementação com GVH aumentou a atividade locomotora nos animais, antes e depois da administração de anfetamina. Da mesma forma, a suplementação com GVH durante a gestação e lactação foi associada ao aumento da atividade locomotora em ambos os ratos jovens e adultos após exposição à anfetamina. Além disso, a suplementação com gordura *trans* ao longo de duas gerações sequenciais de animais também aumentou a locomoção e atividade exploratória em animais expostos ao estresse. Na sequência do estudo, ratas foram suplementadas com óleo de soja/óleo de peixe (OS/OP, razão ótima de ácidos graxos n-6/n-3) ou GVH durante o período da gestação ou lactação e os filhotes adultos foram expostos ao protocolo de estresse crônico leve. Em geral, houve maior incorporação de DHA e ARA durante o período da gestação e LA e ALA durante a lactação e somente a suplementação com GVH permitiu a incorporação de AG *trans* nas membranas neuronais. Além disso, filhotes adultos cujas mães foram suplementadas com GVH mostraram prejuízo na memória de curto e longo prazo antes e após a exposição ao estresse, além de efeitos deletérios sobre marcadores moleculares. Tomados em conjunto, os dados apresentados nesta tese sugerem que uma alimentação saudável durante períodos iniciais do desenvolvimento apresenta efeitos benéficos sobre o SNC, enquanto que o aumento do consumo de alimentos industrializados, os quais são ricos em ácidos graxos *trans*, pode estar envolvido com o desenvolvimento de doenças neuropsiquiátricas, possivelmente em decorrência das alterações na composição fosfolipídica das membranas neuronais.

Palavras-chave: Período perinatal. Azeite de oliva. Gordura *trans*. Marcadores moleculares. Distúrbios neuropsiquiátricos.

ABSTRACT

INFLUENCE OF FATTY ACIDS SUPPLEMENTATION AT DIFFERENT STAGES OF DEVELOPMENT OF RATS: BEHAVIORAL, BIOCHEMICAL AND MOLECULAR ANALISYS

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Fatty acids (FA) are important constituents of the phospholipid membranes and play important roles in the central nervous system (CNS). Evidence suggests that maternal nutrition during early periods of life is directly related to the development of the fetus, newborn and adult since in this period the transfer of long-chain polyunsaturated fatty acids occurs across the placenta and milk, while a inadequate maternal nutrition can alter both morphological and physiological parameters of the puppies. In recent decades, we observed changes in eating habits, which enabled the increased consumption of *trans* fatty acids at the expense of consumption of essential fatty acids, especially the n-3 family. Such changes may affect neuroplasticity and promote the development of neuropsychiatric disorders. Furthermore, frequent situations of stress due to the pressure outside world can also be associated with the development of diseases involving the CNS. In this study, we evaluated the influence of consumption or supplementation of different fatty acids during the perinatal period on behavioral, biochemical and molecular changes in adult rats. From the results presented in the article 1, it was possible to observe beneficial effects from the consumption of a diet enriched with olive oil (20%) at different stages of development, such as pregnancy, lactation or after the weaning of pups to adulthood. In the present study, the body weight and oxidative stress was reduced in all analyzed periods and increased expression of neurotrophic factors such as BDNF and FGF-2 after consumption of diet containing olive oil during the perinatal period. In article 2, it evaluated the relationship between prolonged consumption of hydrogenated vegetable fat (HVF), rich in *trans* fatty acids in different periods of life of rats, and the development of hyperactivity symptoms. The *trans* fat consumption for 10 months and supplementation during pregnancy and lactation over two sequential generations of animals induced active behavior in the forced swimming test. Also, supplementation with HVF increased locomotor activity in animals before and after administration of amphetamine. Similarly, supplementation with HVF during pregnancy and lactation is associated with increased locomotor activity in both young and adult rats after exposure to amphetamine. Furthermore, supplementation with *trans* fat along two sequential generations of animals also increased locomotion and exploratory activity in animals exposed to stress. Following the study, rats were supplemented with soybean oil/fish oil (SO/FO, optimum ratio of fatty acids of the n-6/n-3) or HVF during the period of gestation or lactation and adults offspring have been exposed to mild chronic stress protocol. In general, was observed a greater incorporation of DHA and ARA during the pregnancy period and LA and ALA during lactation and only supplementation with HVF allowed the incorporation of *trans* fatty acids in neuronal membranes. In addition, adults offspring whose mothers were supplemented with HVF showed impairment in short- and long-term memory before and after exposure to stress, as well as deleterious effects on molecular markers. Taken together, the data presented here suggests that a healthy diet during early periods of development has beneficial effects on the CNS, while increased consumption of processed foods, which are high in *trans* fatty acids, may be involved with the development of neuropsychiatric disorders, possibly due to the changes in the phospholipid composition of neuronal membranes.

Keywords: Perinatal period. Olive oil. *Trans* fat. Molecular markers. Neuropsychiatric disorders.

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LISTA DE ABREVIATURAS E SIGLAS

AA	Ácido Araquidônico
AGE	Ácidos Graxos Essenciais
AGMI	Ácidos Graxos Monoinsaturados
AGPI	Ácidos Graxos Poli-insaturados
AGS	Ácidos Graxos Saturados
AGT	Ácidos Graxos *trans*
ALA	Ácido α-linolênico
BDNF	Fator Neurotrófico Derivado do Cérebro
DHA	Ácido Docosahexaenóico
EO	Estresse Oxidativo
EPA	Ácido Eicosapentaenóico
GVH	Gordura Vegetal Hidrogenada
LA	Ácido Linoléico
NFG	Fator de Crescimento Neuronal
OP	Óleo de Peixe
OS	Óleo de Soja
SNC	Sistema Nervoso Central
TDAH	Transtorno do Déficit de Atenção e Hiperatividade

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APRESENTAÇÃO

Esta tese de doutorado está estruturada em seções dispostas da seguinte forma: Introdução, Objetivos, Produção Científica (Artigo 1, Artigo 2 e Manuscrito), Discussão, Conclusão e Referências.

No item **INTRODUÇÃO** e **DESENVOLVIMENTO** encontram-se considerações iniciais sobre o tema desenvolvido nesta tese. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se inseridos nos próprios artigos e manuscrito na seção **PRODUÇÃO CIENTÍFICA** e representam a íntegra deste estudo.

Ao fim, encontram-se os itens **DISCUSSÃO e CONCLUSÃO**, nos quais há interpretações e comentários gerais dos resultados contidos neste estudo.

As **REFERÊNCIAS** referem-se somente às citações apresentadas no item **INTRODUÇÃO, DESENVOLVIMENTO** e **DISCUSSÃO**.

1 INTRODUÇÃO

De acordo com um relatório publicado em 2007, quase 1 em cada 6 pessoas da população mundial sofre de distúrbios neurológicos (UN, 2007). As causas da maioria das doenças neurológicas são vagamente compreendidas e são definidas por numerosos fatores onde a genética e o ambiente desempenham um papel vital e a nutrição, como um fator ambiental, é importante principalmente no desenvolvimento do cérebro (HALLAHAN e GARLAND, 2005).

Os ácidos graxos (AG) desempenham importantes funções na estrutura e função das membranas celulares. O sistema nervoso deve conter níveis equilibrados de ácidos graxos, principalmente aqueles que o organismo de mamíferos não é capaz de sintetizar, denominados ácidos graxos essenciais (AGE) pertencentes às famílias ômega-3 (n-3) e ômega-6 (n-6). Durante o período perinatal os ácidos α-linolênico, linoléico e seus respectivos derivados, o ácido docosahexaenóico (DHA) e ácido araquidônico (AA) são adquiridos através da transferência materno-placentária e leite materno. Após o desmame, os mesmos são obtidos unicamente através da dieta ao longo da vida, onde sua incorporação na estrutura da membrana plasmática confere melhor desempenho das funções cerebrais favorecendo o crescimento neuronal, transdução de sinais, excitabilidade, e expressão de fatores neurotróficos que regulam a sobrevivência e diferenciação celular (BOURRE, 2006; BOUSQUET et al., 2009; BRADBURRY, 2011; QUERQUES, FORTE e SOUIED, 2011).

Ao longo das últimas décadas, ocorreram mudanças qualitativas na nutrição. Hoje, a dieta de uma sociedade industrializada, é caracterizada por um aumento na utilização de gordura vegetal hidrogenada e gordura saturada nos alimentos (BAGGIO e BRAGAGNOLO, 2006) acompanhada por uma significativa redução do consumo de alimentos ricos em n-3 e um aumento na ingestão de ácidos graxos *trans* (AGT) (PFEUFFER e SCHREZENMEIER, 2006). Essa alteração nos hábitos alimentares promoveu um aumento da relação n-6/n-3 de ácidos graxos poli-insaturados (AGPI) (AILHAUD et al., 2006; PATTERSON et al., 2012). Desta forma, o consumo de AGT pode culminar em uma perda de ingestão de ácidos graxos essenciais representando uma perda no valor nutricional dos alimentos, e consequentemente ser considerados fatores de risco para o desenvolvimento de doenças que envolvem o sistema nervoso central (SNC), uma vez que os AGE desempenham um papel funcional importante sobre as membranas biológicas (SARSILMAZ et al., 2003).

A partir da década de 1970, o interesse por interferências ocorridas durante o desenvolvimento fetal começou a se expandir seguindo a “hipótese de Barker” (BARKER e

OSMOND, 1986) que afirma que as influências adversas no início de desenvolvimento e, particularmente, durante a vida intrauterina, pode resultar em mudanças permanentes na fisiologia e no metabolismo, o que pode resultar em aumentado risco de doença na idade adulta (de BOO e HARDING, 2006). Neste sentido, o efeito de alterações ou déficits nutricionais neste período tem sido objeto de diferentes pesquisas (HADDERS-ALGRA, 2011; JANSSEN e KILIAAN, 2014).

Além disso, o estresse também está ligado ao desenvolvimento de doenças do SNC (CHROUSOS, 2009; McEWEN, 2008; SCHMIDT, STERLEMANN e MÜLLER, 2008). Atualmente, é considerado uma adaptação funcional orgânica, que tem como causa principal a pressão do mundo exterior. Embora a base subjacente para a mudança funcional endócrina que ocorre durante o estresse ainda seja desconhecida, existem trabalhos sugerindo que as mudanças na função metabólica, particularmente no metabolismo de ácidos graxos, influenciam mecanismos centrais que regulam as respostas individuais ao estresse (LAUGERO et al., 2002). Além disso, a influência da dieta sobre a manifestação de diferentes tipos de comportamentos induzidos por agentes estressores (FERRAZ et al., 2008) tem sido observada.

A seguir, um breve referencial teórico sobre os principais ácidos graxos constituintes da dieta e sua possível ação sobre as membranas cerebrais. Serão também considerados os efeitos do estresse sobre o SNC, além da interação entre ácidos graxos da dieta e estresse sobre a proteção contra danos cerebrais.

2 DESENVOLVIMENTO

2.1 Ácidos Graxos: Química, classificação geral e principais representantes

Os ácidos graxos (AG) são substâncias encontradas em uma ampla variedade de alimentos e possuem funções estruturais, protetoras e de fornecimento e armazenamento de energia (ALBERTS et al., 2006; COSTA e SILVA, 2002). São formados por uma cadeia hidrocarbonada (2 a 20 ou mais átomos), contendo uma carboxila (COOH) em um extremo da cadeia e uma metila (CH3) na extremidade oposta (MARSZALEK e LODISH, 2005; WALL et al., 2010). A nomenclatura dos ácidos graxos refere-se ao número de átomos de carbono, quantidade e posição das duplas ligações em relação ao grupo metila (GORJÃO et al., 2009). Existe inúmeros comprimentos de cadeia, variando de ácidos graxos de 4 carbonos nos lipídeos de produtos lácteos a ácidos graxos contendo 30 carbonos em alguns lipídios marinhos (LEHNINGER, NELSON e COX, 2002; RATNAYAKE e GALLI, 2009). Quanto à extensão da cadeia, os AG classificam-se em AG de cadeia curta com cauda alifática de menos de 6 átomos de carbono; de cadeia média, com cauda alifática de 6 a 12 carbonos; de cadeia longa, com cauda alifática de mais de 12 carbonos; e de cadeia muito longa, com cauda alifática contendo mais de 22 átomos de carbono. Os AG são classificados também de acordo com o número de insaturações presentes em sua cadeia carbonada (LEHNINGER, NELSON e COX, 2002). Assim, os AG saturados (AGS) não possuem duplas ligações e os insaturados contêm em sua cadeia uma – monoinsaturados (AGMI) - ou mais duplas ligações – poli-insaturados (AGPI) (KIM et al., 2005). As múltiplas duplas ligações de carbono presentes na cadeia dificultam a interação molecular, conferindo característica líquida a esses ácidos graxos a temperatura ambiente, importante propriedade física requerida para manutenção do alto grau de flexibilidade da bicamada lipídica das membranas celulares (CHALON, 2006; HULBERT et al., 2005; INNIS, 2007).

Em mamíferos, incluindo seres humanos, ácidos graxos saturados e monoinsaturados podem ser obtidos pela dieta ou, podem ser sintetizados pela síntese “de novo” de ácido graxo. Pela dessaturação dos ácidos graxos saturados podem ser sintetizados os ácidos graxos monoinsaturados n-9. Essa conversão é realizada pela Δ9 desaturase, que é uma enzima muito ativa em tecidos de mamíferos, que introduz uma dupla ligação entre a posição n-9 – n-10 da cadeia de ácidos graxos (RATNAYAKE e GALLI, 2009). Assim, estes ácidos graxos sintetizados pelo organismo são considerados não essenciais. Os AGS são encontrados em alimentos de origem animal como carne, leite, manteiga, queijo (ácidos palmítico - C16:0 e

esteárico - C18:0), certos vegetais como coco, palma e dendê (ácidos caprílico - C8:0 e cáprico - C10:0) (CARVALHO et al., 2003), assim como os AGMI, os quais também se encontram no azeite de oliva, óleo de canola e de soja e em nozes, sendo o ácido oléico (C18:1) o principal representante da classe (DUNCAN, SCHMIDT e GIUGLIANI, 2004).

Porém, os mamíferos não conseguem sintetizar ácidos graxos com a dupla ligação no carbono n-6 ou n-3. Por esta razão, os AGPI classificados principalmente nas séries ômega 6 e ômega 3, diferenciando-se pela posição da primeira dupla ligação a partir do grupo metílico terminal da cadeia, são considerados ácidos graxos essenciais (AGE) (SIMOPOULOS, 2006; YAQOOB e CALDER, 2007). Dentre os representantes da série n-6, destaca-se o ácido linoléico (C18:2, LA) abundante nos óleos vegetais como de girassol, milho, soja, etc. (McCUSKER e GRANT-KELS, 2010; SANGIOVANNI e CHEW, 2005). O ácido α-linolênico (C18:3, ALA), principal representante da série n-3, é encontrado em peixes marinhos de águas geladas e profundas (sardinha, salmão, cavala, truta, arenque), óleos e produtos derivados de pescados, nozes e óleos vegetais (chia, canola e linhaça) (LARSSON et al., 2004; SOCCOL, HEIDMANN e OETTERER, 2003). Os AG com maior número de carbonos e maior quantidade de duplas ligações como o ácido eicosapentaenóico (EPA, C20:5 n-3) e o ácido docosahexaenóico (DHA, C22:6 n-3), são encontrados tanto nos vegetais (algas, fitoplâncton), quanto nos animais de origem marinha (peixes, crustáceos) (GIBSON, 2004; HULBERT et al., 2005).

Os ácidos graxos *trans* (AGT) são isômeros geométricos e de posição dos AG insaturados naturais e também fazem parte da dieta humana. Produtos de origem animal como carne e leite de animais ruminantes são as principais fontes de AGT, porém, o avanço da industrialização e mudanças na dieta ocidental vem promovendo um considerável aumento no consumo deste tipo de ácido graxo (PADOVESE e MANCINI-FILHO, 2002).

2.2 Ácidos Graxos Poli-insaturados

No fim da década de 20, ao observar alguns sinais e sintomas em pessoas que tinham restrição de gorduras em suas dietas, pesquisadores constataram que havia compostos que eram essenciais para a saúde do organismo (BURR e BURR, 1929) e desde então os ácidos graxos vêm sendo estudados. Alguns AGPI são considerados AGE, pois as células animais não possuem as enzimas dessaturases capazes de especificamente colocar as duplas ligações nas posições n-3 e n-6, de forma que seu suprimento depende unicamente da dieta alimentar (BOURRE, 2004; OKEN e BELFORT, 2010).

Os ácidos linoléico e α-linolênico, quando consumidos, podem ser alongados em cadeias de, pelo menos, 20 ou 22 carbonos (Figura 1). O LA pode ser metabolizado em outros AG da série n-6, incluindo o ácido araquidônico (AA, 20:4 n-6), enquanto o ALA é metabolizado em outros AG n-3, entre eles o EPA e o DHA (McCUSKER e GRANT-KELS, 2010). Este processo metabólico é mediado por enzimas conhecidas como alongases (adição de duas unidades de carbono) e dessaturases (adição da dupla ligação), as quais participam na formação dos AGPI de cadeia longa, resultando em uma competição metabólica entre os dois grupos (BAKER et al., 2012; PERINI et al., 2010; CHILTON et al., 2008).

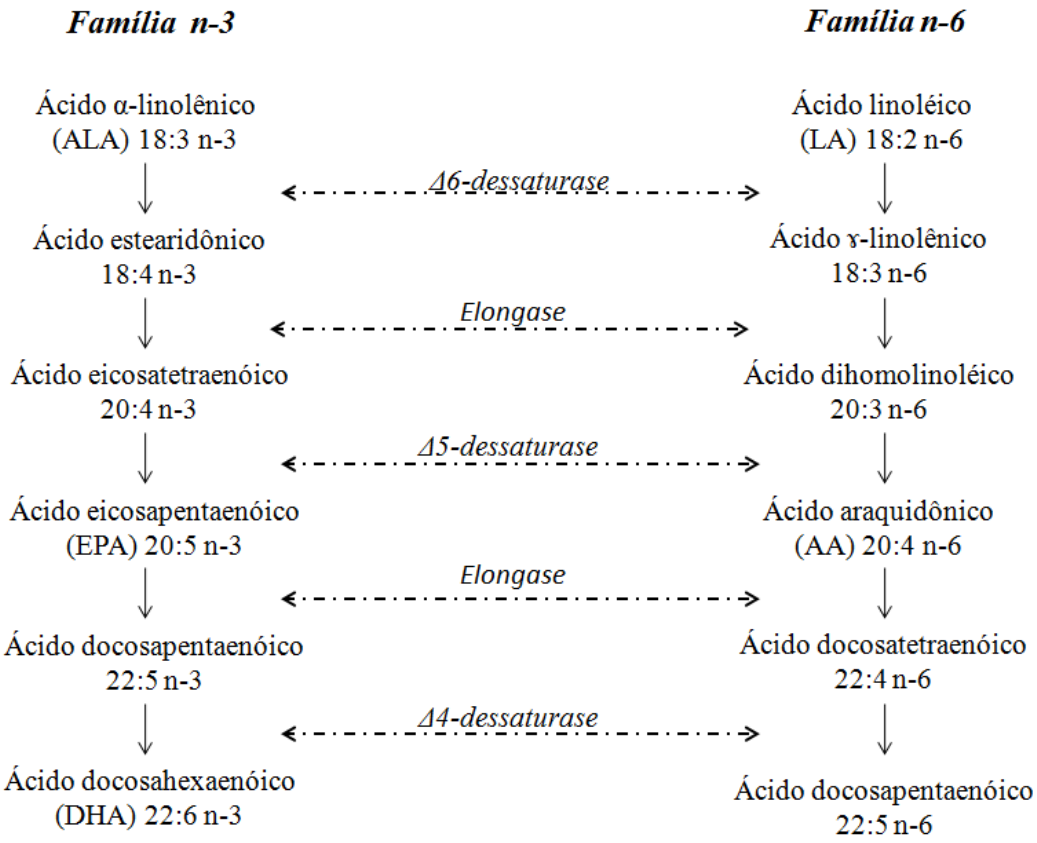

Figura 1. Via metabólica dos ácidos graxos essenciais de cadeia longa (adaptado de LAURITZEN et al., 2001).

Os efeitos benéficos dos AGPI sobre as funções cerebrais parece estar relacionado à sua influência sobre a fluidez da membrana, atividade de enzimas ligadas a membrana, número e afinidade de receptores, produção e atividade de neurotransmissores, além da transdução de sinais que controlam a atividade de neurotransmissores e fatores de crescimento neuronal (McNAMARA e CARLSON, 2006; SUMIYOSHI et al., 2008; BOURRE, 2006; BOUSQUET et al., 2009; BRADBURRY, 2011; QUERQUES, G.; FORTE, R.; SOUIED, 2011).

2.3 Ácidos graxos essenciais e SNC

A literatura possui resultados consistentes que indicam que tanto a parte química, quanto funcional do desenvolvimento e maturação do cérebro pode ser influenciada pela dieta (WAINWRIGHT, 2002). O cérebro necessita de um aporte adequado de ácidos graxos para manter sua integridade estrutural e, consequentemente suas funções normais, principalmente por apresentar a segunda maior concentração de lipídios do corpo, depois do tecido adiposo, representando 36-60% do tecido nervoso (SINCLAIR et al., 2007; UAUY e DANGOUR, 2006).

O DHA é o principal componente dos fosfolipídios das membranas neuronais, abrangendo cerca de 17% do total dos ácidos graxos nesse tecido (HORROCKS e FAROOQUI, 2004; SALEM et al., 2001), sendo um dos grandes responsáveis por aumentar a fluidez da membrana e a plasticidade sináptica e contribuindo para as funções cerebrais (CHEN e SUBBAIAH, 2007; MITCHELL et al., 2003). A presença do DHA nessas estruturas favorece a flexibilidade e a mobilidade das proteínas na bicamada de fosfolipídios, características essenciais para as respectivas funções (BERTRAND, O KUSKY e INNIS, 2006; INNIS, 2007; PONGRAC, SLACK e INNIS, 2007).

Dados experimentais demonstraram que o DHA e o EPA são antioxidantes nutricionais e reduzem a formação de peróxidos de lipídeos no cérebro de ratos (CHOI-KWON et al., 2004) além de proteger ratos jovens contra eventos de excitotoxicidade, como convulsão e isquemia (BAS et al., 2007; STROKIN, SERGEEVA e REISER, 2007). Em humanos, o DHA parece exercer um efeito neuroprotetor, uma vez que baixos níveis deste ácido graxo foram associados com doenças neurodegenerativas, como a doença de Alzheimer (SCHAEFER et al., 2006). A atividade neuroprotetora do DHA também foi evidenciada através de suas propriedades antioxidantes *in vivo* (CALON et al., 2004; HASHIMOTO et al., 2002; YAVIN, BRAND e GREEN, 2002) e *in vitro*, como no aumento da atividade da glutationa redutase (HASHIMOTO et al., 2002), diminuição da oxidação de proteínas (CALON et al., 2004) e dos níveis de peróxidos de lipídios e espécies reativas de oxigênio (HASHIMOTO et al., 2006). Estudos demonstraram ainda que o DHA participa diretamente da modulação da expressão gênica, de processos que envolvem estresse oxidativo, sinalização e divisão celular, crescimento e apoptose (SIMOPOULOS, 2006; YAVIN, 2006). Além disso, DHA e EPA podem modificar a produção e a função de neurotransmissores, tais como a serotonina e a dopamina (DUBOIS et al., 2006; FENTON, HIBBLEN e KNABLE, 2000). Sendo assim, DHA e EPA participam de

numerosas funções celulares, incluindo a fluidez e a atividade enzimática de membrana e síntese de eicosanóides, os quais são essenciais para o desenvolvimento e a manutenção das funções cerebrais (MAZZA et al., 2007).

De particular importância para as funções do SNC, a deficiência dietética e baixos níveis endógenos de ácidos graxos n-3 tem sido associados a um pior prognóstico de doenças psiquiátricas como a esquizofrenia (AMMINGER et al., 2010), depressão (FERRAZ et al., 2008), desordens de hiperatividade (BURGESS et al., 2000), processo de envelhecimento e déficits de aprendizado e memória (BOURRE, 2004). Por outro lado, a suplementação destes ácidos graxos foi benéfica em pacientes com depressão, doença bipolar e esquizofrenia (STOKES e PEET, 2004), capaz de reduzir o estresse oxidativo (EO) em regiões cerebrais críticas, como o corpo estriado (SARSILMAZ et al., 2003) e o hipotálamo (SONGUR et al., 2004), podendo exibir proteção contra parâmetros oxidantes presentes em doenças neurológicas e neuropsiquiátricas.

2.4 Ácidos graxos *trans*

Os ácidos graxos na dieta humana são encontrados naturalmente na configuração *cis*, a qual os átomos de hidrogênio ligados aos carbonos insaturados encontram-se no mesmo plano. Um notável papel desempenhado pela ligação *cis* ocorre nas membranas biológicas constituídas por lipídios, onde o número total de ligações *cis* irá influenciar a sua fluidez. Os ácidos graxos com uma ou mais instaurações na configuração *trans*, ou seja, com os átomos de hidrogênio ligados aos carbonos insaturados em planos opostos, são denominados ácidos graxos *trans* (AGT) (MARTIN et al., 2007). A configuração *trans* resulta em uma conformação molecular linear, similar aos ácidos graxos saturados. Essa conformação mais rígida resulta em diferentes propriedades físicas, tal como ponto de fusão mais alto e melhor estabilidade termodinâmica, associadas às modificações das características químicas sensoriais (REMIG et al., 2010; STENDER, ASTRUP e DYERBERG, 2008) (Figura 2).

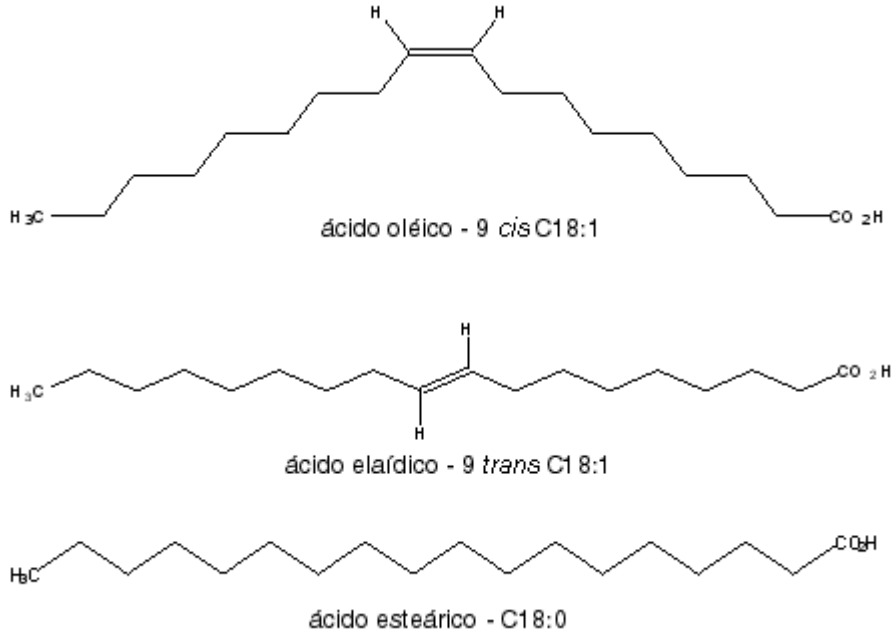

Figura 2. Estrutura química dos ácidos graxos insaturados oléico (*cis*) e elaídico (*trans*), com 18 carbonos, e do ácido graxo saturado correspondente, ácido esteárico (COSTA, BRESSAN e SABARENSE, 2006).

A hidrogenação industrial de óleos vegetais é responsável pela maior parte da ingestão de AGT na dieta humana. Entretanto, AGT também são encontrados naturalmente em baixas concentrações em derivados do leite e gorduras animais, através da hidrogenação biológica no estômago de ruminantes (CRAIG-SCHMIDT, 2006).

No Brasil, a hidrogenação comercial de óleos vegetais data da década de 50, visando à produção de margarinas e gorduras para frituras. Com o desenvolvimento de técnicas de hidrogenação seletiva, os óleos vegetais processados, rapidamente substituíram as gorduras animais na dieta dos brasileiros. Estas gorduras têm sido largamente empregadas na produção de diversos alimentos, como margarinas, coberturas de chocolate, biscoitos, produtos de panificação, sorvetes, massas e batatas "chips", entre outros com o objetivo de dar mais sabor e crocância e principalmente menor rancificação, alongando a vida de prateleira do produto (ECKEL et al., 2007; MARTIN et al., 2005; TARRAGO-TRANI et al., 2006).

Os primeiros estudos relacionando modificações na estrutura molecular dos lipídios com alterações nos seus efeitos biológicos e consequentemente sobre a saúde dos indivíduos foram realizados no ano de 1969. Apesar da sua utilidade tecnológica, os efeitos do consumo desses ácidos graxos nos alimentos têm apresentado grande controvérsia, no que diz respeito ao efeito dos AGT na fisiologia e metabolismo humano.

2.5 Efeitos dos ácidos graxos *trans* sobre a saúde

As primeiras evidências sobre os efeitos do consumo de AGT levaram em consideração os níveis séricos de lipídeos. Estudos sugerem que o consumo elevado desses ácidos graxos pode induzir o aumento nos níveis de LDL-colesterol e triglicerídeos, acompanhado de uma diminuição do HDL-colesterol e consequente aumento da razão colesterol total/HDL-colesterol, os quais são considerados fatores de risco para o desenvolvimento de doenças coronarianas (MENSINK et al., 2003; MOZZAFFARIAN e CLARK, 2009).

As preocupações com os efeitos dos AGT na saúde têm aumentado, uma vez que estes isômeros são estruturalmente similares às gorduras saturadas, modificando as funções metabólicas das gorduras poli-insaturadas e podem competir com os ácidos graxos essenciais em vias metabólicas complexas (MARTIN et al., 2004).

De modo particular, os efeitos de uma deficiência em ácidos graxos essenciais provocados pela incorporação de AGT são importantes principalmente sobre o SNC, no qual os AGPI são constituintes fundamentais. Tem sido observado que animais suplementados com AGT apresentaram proporções elevadas de AG da série n-6 e diminuição de DHA, sugerindo que a composição de AGPI no cérebro é influenciada pelo consumo de AGT (LARQUE et al., 2003). Além disso, os AGT podem ser incorporados em fosfolipídios de membrana, alterando a fluidez, propriedades bioquímicas, e a função das células (SALEM et al., 2001). Especificadamente, uma deficiência em ALA foi capaz de promover déficits de memória, e motores em ratos (WAINWRIGHT, 2002). Níveis reduzidos de AGPI também foram encontrados no cérebro *post mortem* de pacientes esquizofrênicos (HORROBIN et al., 1991; YAO, LEONARD e REDDY, 2000), e prejuízos na neurotransmissão dopaminérgica podem estar relacionadas (KUHN et al., 2015; ZIMMER et al., 1998; 2000).

Ainda, outros estudos mostram que os AGT inibem a reação de dessaturação dos LA e ALA para AA, DHA e EPA, favorecendo o metabolismo de isômeros *trans* incomuns que, se incorporados aos tecidos alteram as funções das membranas (INNIS, 2006). Assim, esta incorporação também pode influenciar mecanismos pró-inflamatórios e pró-apoptóticos através do aumento dos níveis do fator de necrose tumoral, interleucina-6 e proteína C reativa (MOZAFFARIAN et al., 2006), sendo ainda um campo fértil para a pesquisa, considerando que muitas controvérsias ainda persistem quanto ao metabolismo e os efeitos desses ácidos graxos na fisiologia e metabolismo humano.

2.6 Ácidos graxos e nutrição materna

O desenvolvimento e a manutenção das funções psicomotoras e cognitivas, no decorrer da vida, sofrem influência decisiva da nutrição principalmente no período perinatal. Evidências demonstram a importância dos ácidos graxos essenciais, principalmente o DHA para o desenvolvimento cerebral pré-natal, onde participam ativamente na estrutura, função e plasticidade sináptica (INNIS, 2007; SALEM et al., 2001). A presença do DHA durante a gestação e lactação é essencial para a maturação cortical, neurogênese e mielinização, podendo atenuar riscos de prejuízos cognitivos e susceptibilidade às desordens psiquiátricas (BORSONELO e GALDUROZ, 2008; EILANDER et al., 2007; McNAMARA e CARLSON, 2006). Esses ácidos graxos passam da mãe para o feto pela barreira placentária e, após o nascimento, pelo leite materno. Durante o último trimestre intrauterino e os primeiros 18 meses da vida pós-natal humana, e nos primeiros 15 dias após o nascimento em ratos (DOBBING e SANDS, 1979), o DHA e AA são acumulados rapidamente nos fosfolipídios de membrana do sistema nervoso central. Neste momento, o cérebro é especialmente vulnerável a quaisquer deficiências nutricionais, em função de ser o período em que os processos implicados no desenvolvimento cerebral ocorrem com maior rapidez (DIJCK-BROUWER et al., 2005; MORGANE e cols., 2002).

Estudos sugerem que um fornecimento insuficiente de ácidos graxos n-3 durante o desenvolvimento pré e pós-natal diminui o conteúdo de DHA nos tecidos neurais (SCHIEFERMEIER e YAVIN, 2002), o que pode afetar o cone de crescimento dos neurônios, levando a redução da densidade neuronal e arborização dendrítica, em regiões como hipocampo, hipotálamo e córtex (AHMAD et al., 2002; CALDERON e KIM, 2004), podendo assim interferir na liberação de neurotransmissores (CHALON, 2006; ZIMMER et al., 2002). Além disso, pode ocorrer uma variedade de déficits visuais, olfatórios, cognitivos e comportamentais em modelos animais (LIM et al., 2005; MORIGUCHI et al., 2001; NIU et al., 2004). Porém, o suprimento de DHA através do aleitamento materno tem mostrado melhorar o desenvolvimento mental em crianças (HIBBELN, FERGUSON e BLASBALG, 2006).

A relação entre consumo de AGT e a fase perinatal está sendo observada em alguns estudos. Já foi relatado uma correlação inversa entre o peso ao nascer com uma ingestão de isômeros *trans*, sugerindo que os AGT também podem ser transferidos ao feto através da placenta (KOLETZKO e MÜLLER, 1990). Além disso, como os AGT podem inibir as enzimas Δ6 e Δ5 dessaturase, dificultando o metabolismo dos ácidos graxos essenciais, este processo em humanos provoca um impacto na fase gestacional por alterar o desenvolvimento intra-

uterino pela inibição da síntese dos AA e DHA (DECSI e KOLETZKO, 1995), prejudicando a função motora durante o desenvolvimento (BOOYENS e MERWE, 1992).

2.7 Ácidos graxos e neurotrofinas

As proteínas da família das neurotrofinas são fundamentais para o bom funcionamento do sistema nervoso central (CHAO, RAJAGOPAL e LEE, 2006), e são fundamentais para a regulação da sobrevivência neuronal, função e plasticidade sináptica (BRAMHAM, 2008; COHEN e GREENBERG, 2008; REICHARDT, 2006). Níveis aumentados de neurotrofinas tem a função prioritária de proteger os neurônios da excitotoxicidade (LESSMANN, GOTTMANN e MALCANGIO, 2003), portanto as neurotrofinas têm sido descritas como moduladoras da plasticidade sináptica atuando para promover regeneração e reparação do SNC e periférico (LOU et al., 2008; ZHAO et al., 2005). O fator neurotrófico derivado do cérebro (BDNF, *brain derived neurotrophic factor*) e fator de crescimento neuronal (NGF, *nerve growth factor*) são as neurotrofinas mais conhecidas e são sintetizadas a partir de suas proteínas precursoras (pró-neurotrofinas) (CHAO, RAJAGOPAL e LEE, 2006). O BDNF é abundante no tecido cerebral e periférico e suas funções biológicas são mediadas via ligação ao receptor de tirosina quinase B (TrkB), podendo levar a uma variedade de cascatas de sinalização intracelular como a fosforilação do CREB (proteína ligante ao elemento de resposta do AMPc) (BHATIA et al. 2011). A ativação do CREB resulta na expressão de genes envolvidos na sobrevivência neuronal e plasticidade e anormalidades na expressão das neurotrofinas no cérebro estão relacionadas com inúmeras doenças neurológicas (CHAO, RAJAGOPAL e LEE, 2006; DAWBARN e ALLEN, 2003). A ação do NGF através de sua ligação aos receptores de tirosina quinase A (TrkA) (GIGANTE et al., 2003) promovem uma cascata de vias de sinalização independente do receptor (PKC, PI3) (SOFRONIEW, HOWE e MOBLEY, 2001).

Estudos têm mostrado que o mRNA e a expressão das proteínas do BDNF e NGF podem variar conforme o estágio do desenvolvimento. O BDNF está envolvido na neurogênese e sobrevivência neuronal durante o período perinatal (NUMAKAWA et al., 2010), enquanto o NGF atua como fator angiogênico contribuindo para a manutenção, a sobrevivência e função de células endoteliais (NICO et al., 2008), além de estar associado com a ativação de células do sistema imunológico e endócrino (BERRY, BINDOCCI e ALLEVA, 2012). Na idade adulta, o BDNF desempenha um importante papel na modulação da plasticidade sináptica, melhorando assim a aprendizagem e memória (EGAN et al., 2003; HU et al., 2011).

Os ácidos graxos da família n-3, como o DHA, que é um importante componente

estrutural da membrana plasmática (BALOGUN e CHEEMA, 2014; WU et al., 2004) são bem conhecidos por promover a expressão e atividade das neurotrofinas. Estudos recentes sugerem que o estresse oxidativo durante a gravidez ou a deficiência de ácidos graxos n-3 pode reduzir os níveis de DHA que afeta a fluidez das membranas resultando em redução dos níveis e expressão do BDNF e NGF (RAO et al., 2007; SABLE et al., 2011, 2012, 2014), enquanto que a suplementação aumenta a viabilidade das células, por meio da modulação dos receptores de BDNF (KOU, LUCHTMAN e SONG, 2008). Portanto níveis alterados na expressão de BDNF e NGF pode levar ao crescimento e desenvolvimento anormal do cérebro aumentando o risco de doenças neuropsiquiátricas na idade adulta (JOHNSON e MARLOW, 2011; LINDSTRÖM, LINDBLAD e HJERN, 2011).

2.8 Relação entre ácidos graxos e estresse

O estresse é reconhecido em sua cronicidade e identificado como o mal do século XXI**,** segundo a Organização Mundial da Saúde (2010). Suas repercussões estão diretamente ligadas à qualidade de vida do indivíduo, da família e da sociedade. É entendido como um processo complexo e multidimensional em que atuam estressores físicos e/ou psicológicos, sendo definido como um estado de homeostase ameaçado, ou em desequilíbrio, e é controlado por uma série de respostas viscerais e comportamentais, visando à restauração da homeostase do organismo (CARRASCO e VAN de KAR, 2003).

As respostas ao estresse incluem a ativação do sistema nervoso autônomo e do eixo hipotálamo-hipófise-adrenal, a qual acarreta, respectivamente, a secreção de catecolaminas e a liberação de glicocorticóides pelo córtex adrenal (SAPOLSKY, ROMERO e MUNCK, 2000). O aumento sérico dos glicocorticóides pode desencadear alterações biológicas importantes para o organismo, incluindo o desenvolvimento de algumas disfunções psicológicas (McEWEN, 2008, 2010).

Tem sido sugerido também que o estresse pode levar à significativa redução da arborização dendrítica nas regiões do giro do cíngulo, hipocampo, região da amígdala e córtex préfrontal, os quais também predispõem a comportamentos ansiosos e depressivos, demonstrando a influência direta do estresse no sistema nervoso central (LUPIEN et al., 2009).

Recentes evidências demonstram a existência de influência da dieta sobre a manifestação de diferentes tipos de comportamentos induzidos por agentes estressores (FERRAZ et al., 2011; 2008) e o envolvimento dos AGPI na reatividade e sensibilidade do indivíduo ao estresse (McNAMARA e CARLSON, 2006). Estudos têm mostrado que dieta com

óleo de peixe, rica em DHA exerce efeitos anti-estresse (FEDOROVA e SALEM, 2006; ROSS, 2009; TAKEUCHI, IWANAGA e HARADA, 2003) e reduz sintomas depressivos (COLANGELO et al., 2009), possivelmente por reduzir os níveis de glicocorticóides no cérebro através da regulação do eixo do estresse (FERRAZ et al., 2011), sugerindo que a ingestão de AG n-3 pode apresentar efeitos protetores em transtornos psiquiátricos (FREEMAN et al., 2006). Outro recente estudo avaliou a suplementação de AGPI n-3 em pacientes com lesão acidental e mostrou uma redução significativa dos sintomas do transtorno de estresse pós-traumático (MATSUOKA et al., 2010). Estudos com humanos e animais mostra também que a exposição crônica ao estresse é capaz de aumentar a geração de espécies reativas de oxigênio, provocando aumento da peroxidação lipídica no cérebro com consequente dano tecidual (LUCCA et al., 2009; SAHIN e GUMUSLU, 2004; ZAFIR e BANU, 2009).

Ao fornecer uma base fisiológica para a associação entre o metabolismo dos ácidos graxos e doenças do SNC, tem sido sugerido que a desregulação ou deficiência de ácidos graxos podem prejudicar a estabilidade e a composição das membranas neuronais (SU, 2009) levando ao funcionamento anormal do cérebro (HIBBELN et al., 2004). Embora fatores dietéticos sejam os principais responsáveis pelas alterações no equilíbrio e metabolismo dos ácidos graxos, outros, como o estresse pode significativamente contribuir para variação do perfil dos ácidos graxos presentes no organismo favorecendo o desenvolvimento de doenças relacionadas ao SNC.

Sendo assim, os hábitos alimentares durante períodos iniciais do desenvolvimento do sistema nervoso central podem influenciar de maneira significativa o conteúdo de AG das membranas cerebrais. Deve-se considerar ainda a grande incidência de doenças neuropsiquiátricas na população e a influência de fatores estressantes na etiologia destas doenças. Como o mecanismo pelo qual os mesmos causam efeitos deletérios à saúde e sua possível ação sobre as membranas cerebrais não está completamente entendido, mais estudos se fazem necessários, justificando o estudo apresentado nesta tese.

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar a influência do consumo ou suplementação de ácidos graxos poli-insaturados e *trans* durante diferentes períodos de desenvolvimento de ratos sobre parâmetros comportamentais, bioquímicos e moleculares.

3.2 Objetivos Específicos

Protocolo experimental 1:

- ✓ Avaliar a influência do consumo de uma dieta enriquecida com azeite de oliva em diferentes períodos do desenvolvimento de ratos (perinatal e adulto) sobre alterações no status oxidativo e expressão dos fatores neurotróficos BDNF e FGF-2 no córtex pré-frontal e hipocampo.

Protocolo experimental 2:

- ✓ Avaliar a influência do consumo prolongado de gordura vegetal hidrogenada (rica em ácidos graxos *trans*) sobre o desenvolvimento de comportamento hiperativo em ratos.

Protocolo experimental 3:

- ✓ Avaliar a influência da suplementação de uma razão ideal de óleo de soja (rico em ácidos graxos n-6) / óleo de peixe (rico em ácidos graxos n-3) e da gordura vegetal hidrogenada (rica em ácidos graxos *trans*) durante os períodos da gestação ou lactação de ratos, sobre parâmetros comportamentais de memória após exposição ao protocolo de estresse crônico imprevisível;

- ✓ Quantificar o perfil lipídico no hipocampo dos animais adultos nascidos de mães suplementadas com diferentes ácidos graxos durante a gestação ou lactação;

- ✓ Avaliar possíveis alterações nos níveis do BDNF e receptores TrkB no hipocampo dos animais adultos após exposição ao protocolo de estresse crônico imprevisível.

4 PRODUÇÃO CIENTÍFICA

Os resultados inseridos nesta tese apresentam-se sob a forma de artigo 1, artigo 2 e manuscrito, os quais se encontram aqui estruturados. Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se nos próprios artigos, os quais estão dispostos da mesma forma que foram publicados. O manuscrito encontra-se em fase de submissão.

4.1 Artigo 1

OLIVE OIL-ENRICHED DIET REDUCES BRAIN OXIDATIVE DAMAGES AND AMELIORATES NEUROTROPHIC FACTORS GENE EXPRESSION IN DIFFERENT LIFE STAGES OF RATS

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Olive oil-enriched diet reduces brain oxidative damages and ameliorates neurotrophic factor gene expression in different life stages of rats

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Abstract

Our aim was to assess the influence of maternal diet rich in monounsaturated fatty acids on oxidative and molecular parameters in brains of mouse pups as well as their body weight during their lifetime. Female rats received a diet containing 20% of olive oil-enriched diet (OOED) and a standard diet control diet (CD) in different periods: pregnancy, lactation and after weaning until pups' adulthood. On the last prenatal day (Group 1), embryos from OOED group showed smaller body weight, brain weight and lower levels of sulphydryl groups glutathione reduced (GSH) in the brain. On postnatal delay-21 (PND21) (Group 2), pups from OOED group showed higher body weight and brain weight, reduced brain weight/body weight ratio and lower brain lipid peroxidation (LP). On PND70 (Group 3), pups from OOED group showed lower brain LP and higher levels of GSH in prefrontal cortex and lower brain levels of reactive species in the hippocampus. Interestingly, the group of animals whose diet was modified from OOED to CD on PND21 showed greater weight gain compared to the group that remained in the same original diet (OOED) until adulthood. Furthermore, OOED consumption during pregnancy and lactation significantly increased BDNF only, as well as its main transcripts exon IV and VI mRNA levels in the prefrontal cortex. In addition, OOED significantly up-regulated FGF-2 mRNA levels in the prefrontal cortex. These findings open a pioneering line of investigation about dietary adjunctive therapeutic strategies and the potential of healthy dietary habits to prevent neonatal conditions and their influence on adulthood.

Keywords: Antioxidant components; Brain-derived neurotrophic factor; Fibroblast grown factor; Monounsaturated fatty acids; Olive oil-enriched diet

1. Introduction

Morbidity and mortality from chronic diseases in population generally have a multifactorial origin, as the result of the interaction between genetic and environmental factors, being the diet probably the most relevant. Dietary fats are major modifiable environmental factors known to influence growth development and susceptibility to diseases. Different studies have demonstrated the impact of supplementation or deficiency of fatty acids (FAs) on gene expression in the central nervous system (CNS) [1,2]. Although evidence has indicated that disturbances in the uterine environment may program the development of diseases in adulthood [3,4], the action mechanism involved in this fetal programming remains unknown. Several authors have shown that nutritional imbalances during pregnancy cause perturbation during prenatal development, which in turn permanently alters the structure, function and metabolism of tissues and organs [5]. In fact, a nonoptimal fetal environment may be reflected on a smaller body weight at birth and on permanent alterations in the structure and physiology of the body, thus compromising the physical and mental health in later life [6]. While the mechanisms responsible for the long-term effects are not clearly understood, genomic, epigenetic and environmental factors may be taken into consideration [7,8].

Olive oil is the primary fat source in traditional Mediterranean diets [9] and is obtained by mechanical pressing of mesocarps of olives. Olive is a valuable source of monounsaturated (MUFA) and di-unsaturated FA, polyphenol antioxidants and vitamins. The beneficial effects of olive oil are attributed to both MUFA (namely *oleic acid*) content and their antioxidant components: hydroxytyrosol and oleuropein, most of which phenolic in nature. In fact, olive oil and its

Abbreviations: FA, fatty acids; OOED, olive oil enriched diet; CD, control diet; ROS, reactive oxygen species; GSH, glutathione reduced; LP, lipid peroxidation; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; NTF, neurotrophic factors.

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oleic acid contain a number of polyphenols [10], which exert a potentially cardioprotective antioxidant activity [11]. In addition, its consumption was related to the inhibition of platelet function and thromboxane synthesis [12], acting also as antioxidant [13] and neuroprotective [14]. For instance, phenolic compounds from olive oil have been found to decrease reactive species (RS) production, thus eliciting significant free-radical scavenging effects [15,16]. Whereas an analysis with the population showed that high consumption of MUFA and polyunsaturated fatty acids was associated with better cognitive performance in an 8.5-year follow-up [17], a subsequent study has suggested that dietary FA exert a fundamental role on cognitive decline associated to aging or dementia [18]. This protective effect of unsaturated FA was attributed to its influence on the maintenance of the structural integrity of neuronal membranes, affecting their fluidity and neural transmission.

In addition, experimental studies have assessed the influence of dietary factors on plasma or brain levels of the neurotrophin brain-derived neurotrophic factor (BDNF) finding positive correlations between the consumption of n-3 FA, vitamin E and flavonoids with BDNF expression in different animal models [19,20]. In contrast, diets rich in saturated FA and total fat were related to lower brain levels of BDNF, neuronal plasticity and lower cognitive decline [21–23]. Interestingly, some reports regarding fibroblast growth factor (FGF) were related to a classical influence on the development, neuronal repair and learning by different types of structural plasticity, including synaptogenesis and neurite branching [24], thereby regulating the prenatal neurogenesis, as well as the postnatal and adult life [25].

Most research regarding the relationship between diet and genetic risk factors for chronic diseases has derived from observational epidemiological studies. However, studies on dietary intervention represent the most consistent design that is able to provide stronger evidence of causality [26]. Thus, the aim of this study was to treat rats at different stages of life (perinatal and adult) with a diet containing 20% of olive oil, assessing the probability of overweight in adulthood, as well as the susceptibility to the development of oxidative damage in brain areas such as the cortex and the hippocampus. Subsequently, the exposure to this diet from pregnancy until weaning of the pups on the expression of neurotrophic factors was assessed. In fact, these proteins are as important during CNS development as in adulthood, in which they are essential mediators of the neuronal plasticity. On this basis, the influence of dietary FA on the NTF was observed in adult rats submitted to this diet from embryo–fetal period to weaning, *i.e.* during a critical temporal window.

2. Materials and methods

2.1. Animals

All animals were obtained from Harlan Laboratories (Italy). They were kept in plexiglass cages with free access to food and water in a room with controlled temperature (22–23°C), on a 12-h light/dark cycle with lights on at 7:00 a.m., being randomly assigned to different groups according to each experiment.

2.1.1. Experiment 1

Pregnant female Sprague–Dawley (n=30) rats were assigned to one of the two diet groups: standard diet control diet (CD) (n=15) and olive oil-enriched diet (OOED) (n=15) provided as the only food source from pregnancy until the adulthood of pups (Fig. 1). On the last day of pregnancy, one set of dams from each dietary regimen (n=5) was anesthetized (sodium pentobarbital, 50-mg/kg body weight i.p.), euthanized by cervical decapitation, and the brains of embryos from CD (n=5) and OOED (n=5) were removed for biochemical analysis (Group 1). In order to standardize all groups, the embryos were removed 20 days after confirmation of pregnancy by the presence of sperm in the vaginal smear of the dams. Another set of dams from each diet group (n=10) gave birth naturally, and on PND21 (Group 2) or PND70 (Group 3), the pups and adult male rats from CD (n=5) and OOED (n=5), respectively, were anesthetized (sodium pentobarbital, 50-mg/kg body weight i.p.), euthanized by cervical decapitation, and their brains were removed for biochemical analysis, as described below.

2.1.2. Experiment 2

In order to test the effects of olive oil consumption on the body weight of pups, another experimental group of dams (n=10) received a 20% OOED from pregnancy until lactation. After weaning (PND21), half of the male pups remained on the same diet as their mothers (OOED, n=5), and the other half changed to a standard diet (CD, n=5) until PND70. Their body weight was recorded every week during the whole experimental period.

2.1.3. Experiment 3

A separate group of dam rats (n=10) was designated to two experimental groups: standard diet (CD, n=5) and OOED (n=5) being provided as the only food source from pregnancy until weaning of the pups (PND21). After weaning, all the male pups received standard diet (CD) until adulthood (70 days of age). On PND 70, animals were anesthetized (sodium pentobarbital, 50-mg/kg body weight i.p.) and euthanized by cervical decapitation. The prefrontal cortex and the hippocampus were rapidly dissected, frozen on dry ice and stored at −80°C for molecular analysis, as described below.

The standard diet (CD) contained protein 18.5%, FA 3%, fiber 6% and ash 7%; the olive oil enriched diet (OOED) contained protein 17%, FA 20%, fiber 3,5% and ash 4%. The diets were prepared by Mucedola (Milan, Italy) as purified, pelleted rodent diet and stored at refrigeration temperature; fresh diet was provided once a day for further protection against oxidation. The animals were handled according to the guidelines of the National Council for the Control of Animal Experiments, following international norms of animal care and maintenance.

2.2. Biochemical assays

2.2.1. Oxidative stress estimation

2.2.1.1. Lipid peroxidation (LP) levels. LP was determined by the measurement of malondialdehyde (MDA) in brain homogenates. The homogenization was performed with a T25, 18N Ultra-Turrax in potassium phosphate (50-mM)-EDTA (0.1 mM) buffer pH 7.4. LP level was established spectrophotometrically at 532 nm by thiobarbituric acid test employing 0.156 μmol/l/cm as the extinction coefficient and was expressed as nmol MDA/mg wet weight tissue, according to Costa *et al.* [27].

2.2.1.2. RS generation. The RS generation was determined by fluorimetric analysis (Wallac Victor 2 1420 Multilabel Counter, Perkin Elmer, Shelton, CT, USA), according to Lawler *et al.* [28], employing nonfluorescent 2′,7′-dichlorofluorescein (DCF) diacetate (Fluka, Milano, Italy), which is converted into highly fluorescent 2′-7′-DCF in the presence of reactive oxygen species. The DCF procedure is a highly sensitive fluorimetric method that allows the detection of hydroperoxide pmols (excitation, 485 nm; emission, 525 nm). The reactive oxygen species level was expressed as units of fluorescence.

2.2.1.3. Glutathione reduced (GSH) levels. Brain was homogenized 1:5 (w/v) in Tris–HCl buffer (20 mM, pH 7.6). The homogenate was centrifuged at 4°C for 10 min at 9000×g, and the supernatant was centrifuged at 4°C for 1 h at 100.000×g to obtain the cytosolic fraction. The cytosolic supernatant was used for the fluorimetric measurement of GSH content (excitation, 350 nm; emission, 420 nm), according to the method of Hissin and Hilf [29], and expressed as μg/mg protein.

2.2.2. Quantification of mRNAs for neurotrophic factors (BDNF, Isoform IV and VI, FGF-2) by RT-qPCR

For gene expression analyses, total RNA was isolated from the brain regions by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l.; Segrate, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (PCR) to assess mRNA levels of BDNF and basic FGF-2. Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination and subsequently analyzed by TaqMan qRT-PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.) using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories S.r.l.). Samples were run in 384-well format in triplicates as multiplexed reactions with a normalizing internal control (actin). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription), and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for annealing and extension reactions. A comparative cycle threshold method was used to calculate the relative target gene expression. Probe and primer sequences used were purchased from Applied Biosystem Italia. For graphic clarity, data were calculated and presented as means percent of control group.

2.3. Statistical analysis

The data were analyzed using independent Student's t-test. All of the data were expressed as means±S.E.M. P<0.05 was regarded as statistically significant.

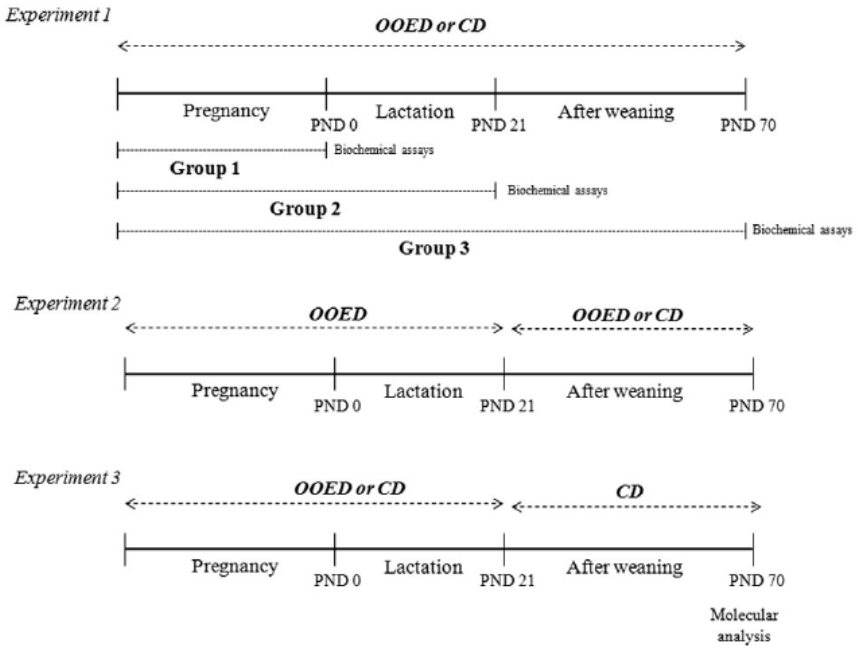

Fig. 1. Experimental design. Diet enriched with 20% OOED or a standard diet (CD) in different periods of development.

3. Results

3.1. Influence of a diet enriched with 20% olive oil (OOED) or a standard diet (CD) on body and brain weight (Table 1 and Fig. 2)

In Experiment 1, embryos (Group 1) from OOED group showed smaller body and brain weight, which were reflected on the decreased brain weight/body weight ratio in comparison to CD group ($P<0.05$; Table 1). On PND21 (Group 2), OOED supplementation showed higher body and brain weight, while the brain weight/body weight ratio remained lower in relation to CD group ($P<0.05$; Table 1). In adulthood (Group 3), animals from both OOED and CD groups showed similar body weight and brain weight (data not shown). In Experiment 2, interestingly, animals whose diet was changed from OOED to CD on PND21 showed increased body weight in relation to those that remained with the same OOED diet until adulthood (Fig. 2).

Table 1
Mean values of body weight, brain weight, brain weight/body weight ratio, glutathione (GSH) and LP levels (MDA) of rats treated with a 20% OOED or a standard diet (CD) on embryo–fetal period (Group 1) and pregnancy until weaning period (Group 2).

		CD	OOED
Group 1	Body weight (g)	4.00±0.04	3.80±0.08 *
	Brain weight (g)	0.15±0.03	0.13±0.00 *
	Brain weight/body weight ($g\times10^{-2}$)	3.91±0.09	3.42±0.11 *
	RS	12.60±0.33	12.73±0.62
	GSH	1.61±0.27	0.69±0.20 *
Group 2	Body weight (g)	47.09±1.83	57.00±1.74 *
	Brain weight (g)	1.289±0.03	1.37±0.01 *
	Brain weight/body weight ($g\times10^{-2}$)	2.760±0.11	2.42±0.06 *
	RS	44.68±1.30	41.52±1.10
	GSH	1.98±0.05	1.87±0.06
	LP	53.06±0.87	46.07±1.54 *

Data are expressed as mean±S.E.M.
* Indicates significant difference between standard diet and OOED ($P<0.05$).

3.2. Influence of a diet enriched with 20% olive oil (OOED) or a standard diet (CD) on the oxidative stress markers (Table 1 and Fig. 3)

Embryos of Group 1 and pups of Group 2 showed no differences in RS generation, which was quantified in the total brain (Table 1). Nevertheless, an increased GSH level was observed in the total brain of embryos from CD group ($P<0.05$; Table 1), but this effect was not observed in pups (Table 1). In adulthood, OOED group showed lower levels of LP ($P<0.001$; Fig. 3A) and RS generation ($P<0.001$; Fig. 3B) in the prefrontal cortex and the hippocampus, respectively. In addition, this same experimental group showed higher GSH level ($P<0.001$; Fig. 3C) in the prefrontal cortex, in comparison to CD group.

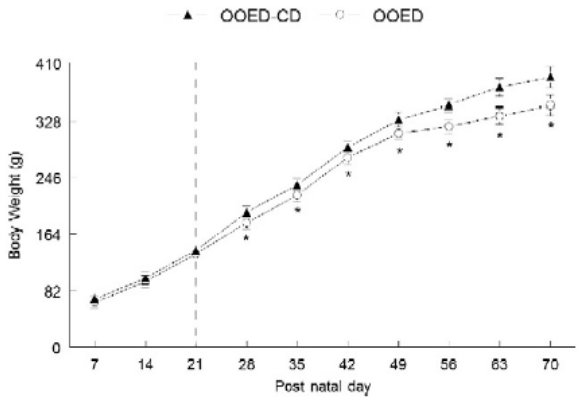

Fig. 2. Effect of a diet enriched with 20% OOED from pregnancy until adulthood in comparison with animals whose diet was changed from OOED to CD from PND21 until adulthood (OOED-CD) (Experiment 2). Data are expressed as mean±S.E.M. *Indicates significant difference between diet–control and diet group ($P<0.05$).

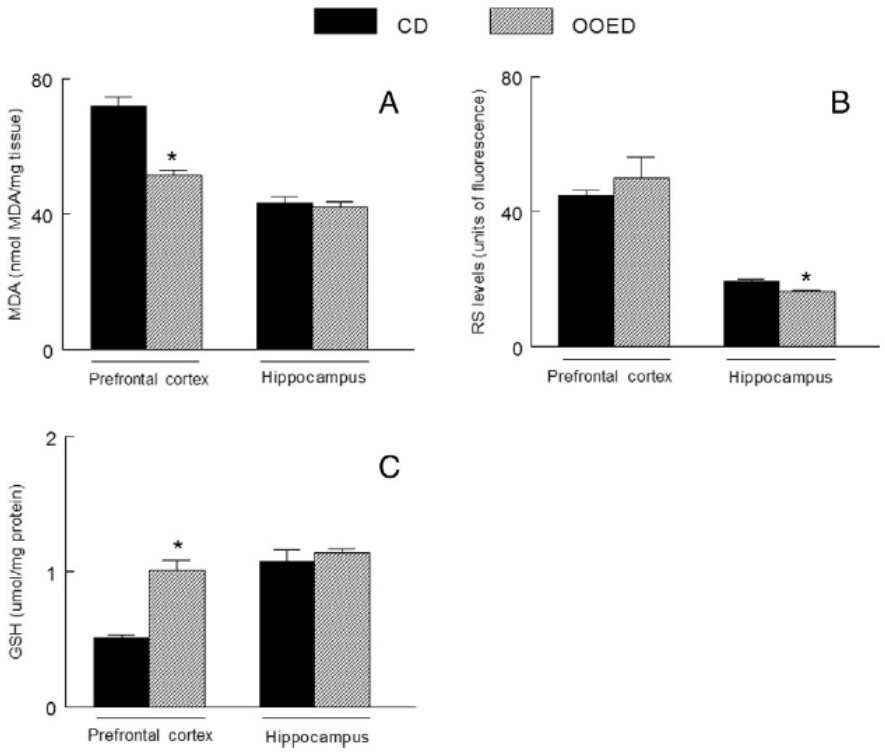

Fig. 3. Effect of a diet enriched with 20% OOED or a standard diet (CD) from pregnancy until adulthood rats (Group 3) on levels of MDA (A), RS (B) and GSH (C) in prefrontal cortex and hippocampus. Data are expressed as mean±S.E.M. *Indicates significant difference between standard diet and diet enriched with olive oil ($P<0.05$).

3.3. Influence of a diet enriched with 20% olive oil (OOED) or a standard diet (CD) on neurotrophic factor gene expression (Fig. 4)

In Experiment 3, the OOED supplementation significantly increased BDNF mRNA levels in the prefrontal cortex ($P<0.05$; Fig. 4A), although not in the hippocampus. OOED consumption was also associated with an up-regulation of BDNF exon IV and exon VI mRNA levels, two main neurotrophin transcripts, when compared with CD group ($P<0.001$, Fig. 4B and C). Interestingly, OOED significantly up-regulated FGF-2 mRNA level in both the prefrontal cortex and the hippocampus in relation to CD group, although the effect reached statistical significance only in the prefrontal cortex ($P<0.001$; Fig. 4C).

4. Discussion

The traditional Mediterranean diet includes a considerable proportion of vegetables, cereals, fruit, fish and mainly olive oil. Some studies have shown that high monounsaturated fat intake seems to be associated to a reduced risk of aging-related cognitive decline [30] as well as protection against Alzheimer's disease [31]. In fact, this beneficial influence has been attributed to the type of FA present in olive oil, which may contribute to the maintenance of the structural integrity of neural membranes [30,31]. Also, there is a lower incidence of cancer and heart diseases in Mediterranean regions [32,33]. From these considerations, the aim of our study was to assess the influence of olive oil consumption at different life stages on the probability of overweight in adulthood and during the developmental period, as well as its influence on oxidative parameters and gene expression in different brain areas.

Experimental studies have shown that the amount of dietary fats is related to behavioral and oxidative damage, also impairing the neurodevelopment of animals [34–36]. In addition, recent reports from our group have shown a significant relationship between behavioral and molecular damage to the CNS and the type of dietary FA, with no quantitative influence since the diets had the same fat content [37–40], indicating that the type of FA is able to exert a key role on cellular functions. In this sense, although the diets used in the current study present different fat amounts, we believe that the beneficial influence of OOED observed result from the FA present in olive oil, with no influence of fat amount.

Our findings showed that OOED consumption could exert a significant influence on body weight of pups, depending on the diet period. On the other hand, the change from OOED to CD after weaning could increase the body weight in adulthood, whereas this increase was not observed when OOED was maintained during the whole period. These findings are in fact interesting, since several studies have shown an inverse relationship between the Mediterranean diet and overweight or obesity [41,42]. They are consistent with other literature data, which showed that the Mediterranean diet could decrease fat mass and increase lean mass [43]. Similarly, subjects treated with a Mediterranean-style diet rich in olive oil showed lowest body weight gain after 3 years of intervention [44]. The fact that a higher consumption of olive oil facilitates the maintenance of body weight was also reported in a large Spanish cohort study by Bes-Rastrollo *et al.* [45] in which the authors found that higher olive oil consumption was associated with a lower likelihood of weight gain. This beneficial effect may be due to the presence of MUFA in olive oil, which can intervene actively on body weight regulation. It has already been demonstrated in different studies that MUFA act on: (a) regulation of the appetite [46]; (b) intestinal absorption of fats [47]; (c) lipolytic activity of adipocytes [48]; (d) thermogenesis [49], among other functions.

In addition, it has been suggested that the beneficial effects of olive oil in the prevention of chronic diseases are attributed to a favorable FA profile and to the antioxidant potential of its polyphenols [9,50], besides the presence of vitamin E [51]. Experimental studies [52,53]

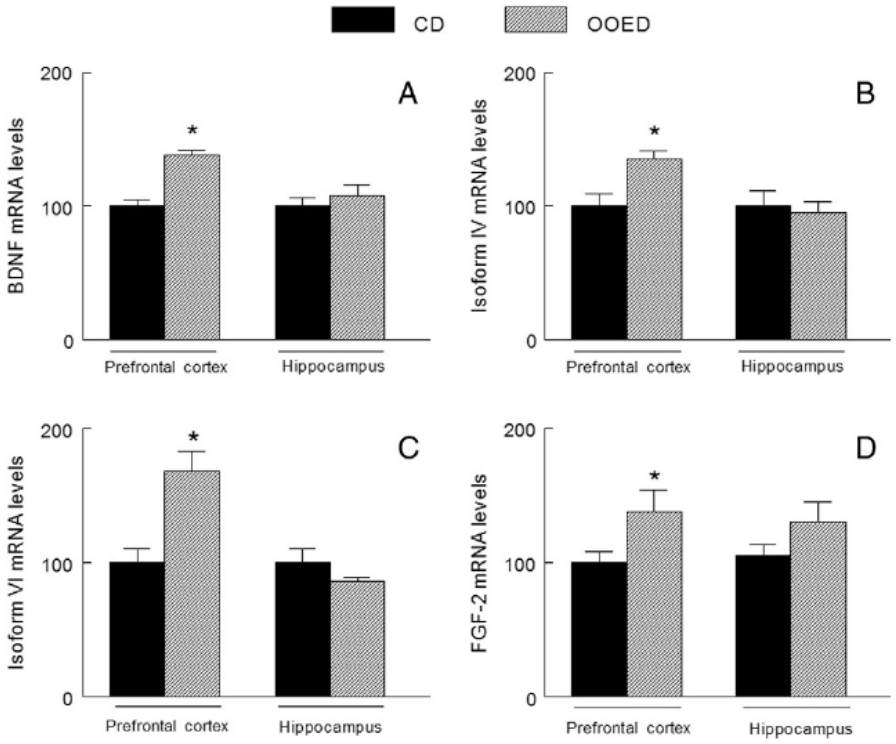

Fig. 4. Effect of a diet enriched with 20% OOED or a standard diet (CD) from pregnancy until weaning (Experiment 3) on BDNF, isoform IV, isoform VI and FGF2 mRNA levels in prefrontal cortex and hippocampus of adult rats. Data are expressed as mean±S.E.M. *Indicates significant difference between standard diet and diet enriched with olive oil ($P<0.001$).

have demonstrated that phenolic compounds present in olive oil such as tyrosol, hydroxytyrosol and oleuropein can exert antioxidant, antiinflammatory and gene-regulatory activities. In addition, these phenolic compounds may reduce superoxide and hydroxyl radicals, thus inhibiting the respiratory burst of neutrophils and related radicals [54,55]. Furthermore, De La Puerta *et al.* [13] showed that polyphenols in olive oil inhibit *in vitro* activity of leukocyte 5-lipoxygenase and the formation of RS in these cells. The current findings are in accordance with those, since rats provided with OOED had lower levels of LP in total brain at weaning, lower levels of LP and decreased RS generation in the cortex and the hippocampus, respectively, as well as increased GSH levels in the cortex of adult rats. Previous studies showed that a diet rich in saturated FA may lead to changes in the oxidative damage in different brain areas, which are basically observed by increased LP together with decreased antioxidant defenses, whereas dietary olive oil could reverse those parameters [56], as observed here. Some reports showed that n-3- or n-6-enriched diet results in less production of lipid peroxides in both brain and liver [57,58]. In fact, it is suggested that the effect of n-6 FA may result from higher antioxidant defenses in the brain [59]. In the current study, rats provided with olive oil, which also contains n-6 polyunsaturated FA, showed results compatible with those studies. The present findings show that a diet rich in olive oil could reduce oxidative damage in brain areas by not only decreasing LP levels and RS generation, but also by enhancing the antioxidant defense system, estimated here by GSH levels.

We believe that our findings have considerable relevance, mainly because most age-related diseases have been associated with high-grade inflammation triggered and sustained by oxidative damage. This relationship has been inferred from observational studies in which adequate antioxidant content in the diet was associated with lower rates of chronic disease [60]. In fact, different authors have shown the beneficial influence of dietary olive oil [61], mainly because MUFA-rich membranes are more resistant to oxidative processes, protecting the aged cell [62] and the integrity of mitochondrial structure. Furthermore, stability of DNA against oxidation was also enhanced when rats had an OOED [63].

In addition, our findings show that, in comparison with CD group, exposure to OOED in early life leads to increased BDNF gene expression in the prefrontal cortex of adult rats, which may be sustained by enhanced transcription by exon IV and exon VI promoters. Moreover, as BDNF has been related to several actions such as synaptic plasticity, neuronal survival and differentiation, its gene expression has been reported to be associated with different neurodegenerative or psychiatric disorders [64–66]. In fact, the intake of omega-3 FA [19,67] showed a positive correlation with brain BDNF levels as well as the intake of vitamin E [68] or flavonoids [20]. Some authors have shown that antioxidant compounds present in olive oil can also alter the BDNF levels in different brain areas [69,70]. In fact, these antioxidant compounds can produce an up-regulation of BDNF levels [22,71], what is in line with our findings, since olive oil consumption could increase BDNF gene expression in the prefrontal cortex of rats. In this sense, BDNF gene itself is complex and may be expressed by four different mRNA isoforms, which can be regulated by different signaling cascades. In some studies, BDNF exons I and IV were transcriptionally up-regulated during the consolidation of fear learning [72,73]. Another study showed that only BDNF exon IV is transcriptionally up-regulated in the hippocampus during the consolidation of fear learning, suggesting that BDNF exon usage is brain region specific and responds differently to environmental cues [74]. However, it is well established that there is a decrease in BDNF mRNA levels [75,76] in animals with cognitive impairment [77]. Here, we did not directly test learning, but it has been demonstrated that rapid changes of hippocampal BDNF occur in contextual learning [78], and

its increased expression in fear conditioning is attributed to the modulation of exon IV [79]. On this basis, we may speculate that the significant elevation of exon IV and VI in OOED group might facilitate learning mechanisms in adulthood [80].

Consistent with the growing number of identified FGFs and the important role of these trophic factors in the CNS for their classical influence on neuronal development, repair and protection, as well as learning and neuronal plasticity [24], we also measured the mRNA levels of FGF-2 after dietary supplementation with olive oil. FGF-2, the prototype member of the FGF family, is well known to mediate several types of structural plasticity, including synaptogenesis and neurite branching [24], and to regulate prenatal as well as postnatal and adult neurogenesis, by inducing the proliferation of neuronal progenitor cells in the hippocampus and the subventricular zone [25]. According to some authors, FGFs are neuroprotective, and the reduced expression of trophic factors belonging to the FGF family could increase the vulnerability of selected neuronal populations, which characterize many neurological and psychiatric disorders [81]. For example, it has been demonstrated that major depressive and bipolar disorders are associated with a significant reduction of FGF-transcripts (including FGF-2) at the fronto-cortical level, as well as FGF receptors, in brain areas compared with healthy subjects [82]. The current study showed that lifelong consumption of olive oil may increase FGF-2 mRNA expression. These data are in line with other studies, which demonstrated a prominent role for FGFs and FGF receptors at different stages of brain development [24], indicating that events that interfere with the correct expression of the FGF system might have permanent effects on the CNS function. Given the reduction of the cerebral expression of several members of the FGF family observed in depressed subjects [83,84], our finding on the impact of olive oil on FGF-2 expression may be crucial. Indeed, based on the well-established function of BDNF and FGF-2 on neuronal plasticity, it may be inferred that nutritional interventions during critical developmental windows produce enduring effects on the brain function as well as an enhanced resilience toward pathologic conditions. Moreover, the Mediterranean diet is powerfully antioxidant, and a number of international scientific organizations now recommend preventing conditions in which oxidative stress may play an etiological role [85]. In fact, olive oil producers and the food industry are encouraged to establish means to increase the phenolic content of olives, as well as their derived products.

In summary, our data suggest that exposure to OOED throughout life may produce persistent changes on the oxidative status of the brain cortex and the hippocampus, as well as on trophic molecules expression, especially in the prefrontal cortex. These findings open a new line of investigation regarding dietary adjunctive therapeutic strategies and the potential of healthy dietary habits to prevent neuropsychiatric and/or neurological conditions. To assist in addressing the global crisis around chronic neuropsychiatric disorders and neurodegenerative conditions, which are increasing in young and elderly, respectively, preventative measures are required in the search of synergistic properties of nutrients. Accordingly, the Mediterranean diet, which includes olive oil consumption, is able to act favorably on both cerebral oxidative status and trophic factors, thus contributing favorably to decrease the incidence and severity of these neuropsychiatric conditions.

Author contributions

C.S.P, A.M.T., F.C., R.M. and S.F. were responsible for acquisition, analysis and interpretation of data and drafting the article; K.R. and V.T.D reviewed it critically for intellectual content of the study; M.E.B, A.E.P and M.A.R contributed effectively to conception and design of the study and final approval of the version to be submitted.

Conflict of interest

The authors declare that there are no conflicts of interest.

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4.2 Artigo 2

CHRONIC CONSUMPTION OF *TRANS* FAT CAN FACILITATE THE DEVELOPMENT OF HYPERACTIVE BEHAVIOR IN RATS

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Chronic consumption of *trans* fat can facilitate the development of hyperactive behavior in rats

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HIGHLIGHTS

- Consumption of foods rich in *trans* fatty acids (TFA) is growing in Western countries.
- TFA for 10 months and across two generations induced active coping in forced swimming task.
- TFA was associated with increased locomotion before and after amphetamine administration.
- TFA across two generations increased locomotor and exploratory activities after environmental stress.
- Processed foods in early life may facilitate the development of hyperactive-like symptoms.

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ABSTRACT

In recent decades, the increased consumption of processed foods, which are rich in hydrogenated vegetable fat (HVF), has led to a decreased consumption of fish and oilseed, rich in omega-3 fatty acids. This eating habit provides an increased intake of *trans* fatty acids (TFA), which may be related to neuropsychiatric conditions, including inattention and hyperactivity. In this study, we evaluated the potential connection between prolonged *trans* fat consumption and development of hyperactivity-like symptoms in rats using different behavioral paradigms. *Trans* fat intake for 10 months (Experiment 1), as well as during pregnancy and lactation across two sequential generations of rats, (Experiment 4) induced active coping in the forced swimming task (FST). In addition, HVF supplementation was associated with increased locomotion before and after amphetamine (AMPH) administration (Experiment 2). Similarly, HVF supplementation during pregnancy and lactation were associated with increased locomotion in both young and adult rats (Experiment 3). Furthermore, *trans* fat intake across two sequential generations increased locomotor and exploratory activities following stressors (Experiment 4). From these results, we suggest that chronic consumption of *trans* fat is able to enhance impulsiveness and reactivity to novelty, facilitating hyperactive behaviors.

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is a serious neuropsychiatric condition that affects about 3–7% of school-aged children [1] and approximately 4% of adults [2,3]. ADHD is characterized by a cross-situational pattern of inattention, hyperactivity, and/or impulsivity, interfering with appropriate social and/or academic functioning [4]. Studies have investigated the association between reduced intake of n − 3 PUFA and inattention, hyperactivity and behavioral disorders [5,6]. Some authors have proposed that ADHD may be related to deficiencies in the conversion of EFA to its long chain derivatives (LC-PUFA) [7], which are particularly important in the neural membrane structure, exerting beneficial influences on signals transduction of the normal brain, thus affecting emotional functions [8,9].

In animal models of ADHD, locomotor hyperactivity is the main outcome measured and several reports have revealed an increase in locomotion in animals fed n − 3 PUFA deficient diets [10–12]. Interestingly, the exposure of mice to n − 3 PUFA deficient diet during pregnancy was able to increase locomotor activity in the offspring [13]. Blood biochemical evidence has suggested that a relative deficiency in n − 3 PUFA (in serum,

Abbreviations: ADHD, attention-deficit hyperactivity disorder; AMPH, amphetamine; AS, acute stress; DHA, docosahexaenoic acid; EFA, essential fatty acids; FA, fatty acids; FST, forced swimming task; HVF, hydrogenated vegetable fat; LC-PUFA, long chain polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids; OFT, open-field task; SO, soybean oil; TFA, *trans* fatty acids.

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plasma and cell membrane) may underlie some of the behavioral and learning problems central to ADHD [14–16]. Likewise, spontaneously hypertensive rats exhibit locomotor hyperactivity together with low levels of DHA in plasma and brain membranes [17,18], signalizing the involvement of brain FA content in the development of hyperactive symptoms.

Characteristic of modern society, changes in dietary habits such as a high consumption of processed foods, especially fast food rich in *trans* fatty acids (TFA) [19], can be considered a risk factor for the development of central nervous system diseases [20–22]. In recent decades, there has been an increased presence of hydrogenated vegetable fat and saturated fat in foods [23] accompanied by a significant reduction in the consumption of foods rich in essential fatty acids (EFA) [24]. Regular consumption of TFA may eventually result in a loss of EFA, with unpredictable impacts on human health, because TFA derivatives may be incorporated into membrane phospholipids [25] and alter membrane fluidity, plasticity and neurotransmission [26,27]. *Trans* fat intake has also been linked to cognitive dysfunction [28,29], changes in dopaminergic neurotransmission [30], addiction [31], mania [32], movement disorders [25] and higher sensitivity to stress and anxiety [33].

So far, most studies about hyperactivity have been focused on dietary omega-3 fatty acids deficiencies [34–36], but little is known about the influence of long-term chronic consumption of TFA, especially in western countries, on the development of hyperactive behaviors. Our hypothesis of a link between regular consumption of *trans* fat and hyperactivity-like behavior emerged from unexpected behavioral findings from several research studies using different experimental paradigms, whose initial aim was not hyperactivity itself. These behavioral observations were so significant that we thought they could not be ignored and deserved a separate report.

2. Material and methods

The experiments were conducted with male Wistar rats from the central animal breeding facility of the Universidade Federal de Santa Maria (UFSM), RS-Brazil, kept in Plexiglas cages with free access to food and water in a room with controlled temperature (23 °C ± 1 °C) and on a 12 h-light/dark cycle with lights on at 7:00 a.m. All the experimental protocols were approved by the Animal Ethical Committee of this university (24/2010; 23/2010; 004/2012), which is affiliated to the Council for Control of Animal Experiments (CONCEA), following international norms of animal care and maintenance.

2.1. Experiment 1

The trial was conducted with 20 male Wistar rats weighing 40–60 g at the beginning of the treatments. Immediately after weaning (21 days of age), rats were randomly assigned to two experimental groups (n = 10) and the diets started. Dietary supplementation consisted in the incorporation (20%) [25,37] of soybean oil (SO) (rich in polyunsaturated fatty acids-PUFA) or hydrogenated vegetable fat (HVF) (rich in *trans*-monounsaturated and saturated-TFA) in standard chow, Supralab® (Alisul alimentos LTDA, São Leopoldo, RS Brazil) as purified pelletted rodent diet and stored at refrigeration temperature (Tables 1 and 2). SO was considered as a control group (C-SO), mainly because it contains adequate levels of PUFA, n − 6/n − 3 ratio within acceptable limits [38–40], and by its elevated consumption worldwide [25,37]. The two experimental groups (C-SO and HVF) were isocaloric in order to prevent metabolic differences between animals of different experimental groups [41,42] from interfering with the antioxidant defense system [43] and dopamine and serotonin neurotransmission [44]. The consumption of the diets was monitored every other day. Both experimental groups (C-SO and HVF) were submitted to behavioral observations in the forced swimming task (FST) from ten months of dietary consumption.

Table 1
Composition of the diet.

Ingredient	Amount (g/kg diet)
Casein	180
Cornstarch	460
Sucrose	230
Cellulose	20
Fat[a]	20
Mineral mix[b]	50
Vitamin mix[c]	10

[a] Represented by soybean oil or hydrogenated vegetable fat.
[b] Composition (g/kg): sucrose 110.7; $CaCO_3$, 240; K_2HPO_4, 215; $CaHPO_4$, 215; $MgSO_4.7H2O$, 100; NaCl, 60; MgO, 40; $FeSO_4.7H_2O$, 8; $ZnSO_4.7H_2O$, 7; $MnSO_4.H_2O$, 2; $CuSO_4.5H_2O$, 1; $Na_2SiO_7.3H_2O$, 0.5; $AlK(SO_4)_2.12H_2O$, 0.2; K_2CrO_4, 0.15; NaF, 0.1; $NiSO_4.6H_2O$, 0.1; H_2BO_3, 0.1; $CoSO_4.7H_2O$, 0.05; KIO_3, 0.04; $(NH_4)6Mo_7O_{24}.4H_2O$, 0.02; LiCl, 0.015; Na_2SeO_3, 0.015; NH_4VO_3, 0.01.
[c] Composition (g/kg): sucrose, 549.45; retinyl acetate, 1; cholecalciferol, 0.25; DL-a-tocopheryl acetate, 20; phylloquinone, 0.1; thiamin HCl, 1; riboflavin, 1; nicotinic acid, 5; calcium pantothenate, 2.5; pyridoxine HCl, 1; biotin, 1; folic acid, 0.2; cyanobalamin, 2.5; cholin HCl, 200; DL-methionin, 200; *p*-aminobenzoic acid, 5; inositol, 10.

Table 2
Fatty acid composition of diets enriched with different fats (% of total fatty acids identified).

Fatty acids	Soybean oil	Hydrogenated vegetable fat
∑SFA	19.36	25.83
∑MUFA	28.56	59.10
∑ n − 3 FA	4.72	0.67
∑ n − 6 FA	46.47	13.69
∑PUFA	51.19	14.36
∑*trans* FA	0.61	16.51

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

2.2. Experiment 2

A trial was conducted with 32 male Wistar rats weighing 30–40 g at the beginning of the study. Twenty-one-day-old rats were assigned to two experimental groups (n = 16): 0.1% soybean oil (SO) and 0.1% hydrogenated vegetable fat (HVF) (Table 3). SO and HVF were incorporated to tap water as a homogenous 1% Tween suspension [45], which was prepared daily and offered to the animals in place of drinking water in dark bottles. The consumption was monitored daily and no differences between the experimental groups were observed (data not shown). After 8 weeks of *ad libitum* intake of FA, half of each experimental group (n = 8) received a single daily injection of amphetamine (4 mg/kg/ip) for 8 consecutive days [46]. Two hours after the last AMPH administration, the locomotor activity was evaluated in the open field task (OFT).

Table 3
Fatty acid composition of different dietary supplementation (% of total fatty acids identified).

Fatty acids	Soybean oil	Hydrogenated vegetable fat
∑SFA	18.07	25.94
∑MUFA	26.03	43.35
∑ n − 6 FA	50.25	17.89
∑ n − 3 FA	5.48	0.48
∑ PUFA	55.73	18.37
∑*trans* FA	0.15	19.79

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 4
Fatty acid composition of different dietary supplementation (% of total fatty acids identified).

Fatty acids	Soybean oil	Hydrogenated vegetable fat
∑ SFA	10.50	15.80
∑ MUFA	34.00	43.41
∑ n − 6 FA	49.00	27.78
∑ n − 3 FA	4.95	1.84
∑ PUFA	54.50	29.45
∑ *trans* FA	0.40	11.82

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

2.3. Experiment 3

One week before mating, virgin female adult Wistar rats were supplemented (3 g/kg; p.o.) [47] with soybean oil (SO) or hydrogenated vegetable fat (HVF) (Table 4) and maintained under the same supplementation during pregnancy and lactation. From weaning (postnatal day 21), one male pup from each litter (n = 8) and the same supplementation was grouped and kept under the same oral treatment until 40 or 90 days of age. At both these times, rats received a single daily injection of amphetamine (AMPH, 4 mg/kg/ip) for 8 consecutive days and submitted to behavioral observations in the open-field task (OFT).

2.4. Experiment 4

Pregnant female Wistar rats were randomly assigned to two experimental groups (n = 7 for each group): Control-soybean oil (C-SO, rich in PUFA) and HVF (rich in TFA), which were supplemented daily by oral gavage with 3 g/kg body weight [47] (Table 4) from conception until weaning. One female pup of each litter was maintained on the same supplementation until adulthood, when they were mated. These dams were kept on the same original supplementation until weaning of the litter of the second generation, when the pups were supplemented until 40 days of age. At day 41, adolescent male rats of each supplemented group (n = 7) were exposed to the acute restrain stress (AS) procedure [48]. After 24 h of AS exposure, animals were submitted to behavioral assessments. For all experiments, SO and HVF were purchased in a local supermarket.

2.5. Behavioral evaluations

2.5.1. Forced-swimming task (FST)

Detailed procedures of the FST were described elsewhere [49–51] (Porsolt et al., 1978; Detke et al., 1995; Detke and Lucki, 1996). Briefly, the rats were placed in vertical Plexiglass cylinders (40 cm high and 20 cm in diameter), containing water (25 °C) 30 cm deep. They were placed into the water for a 15-min period (pretest session). At the end of this pretest phase, each rat was removed from the water, partially dried with a towel, and placed in a plastic cage illuminated with a heat lamp. Twenty-four hours later, the rats were exposed to the same experimental conditions outlined above for 5 min (test session). The immobility, climbing and swimming time were recorded and then rated by two trained raters, who were blind to the dietary treatment. Immobility was considered as no additional activity other than that required to keep the head above water, climbing was defined as upward struggling movements of the forepaws at the side of the cylinder, and swimming as movement around the cylinder.

2.5.2. Open-field task (OFT)

In this test the rats were placed individually at the center of an open field arena (40 × 40 × 30 cm) with black plywood walls and white floor divided into nine equal squares, as previously described by Kerr et al. [52]. The number of square crossings (horizontal squares crossed) and rearings (vertical movements) were recorded for 5 min under dim light (30 lx) [53] and used as measures of spontaneous locomotor activity and exploratory behavior, respectively.

2.6. Statistical analysis

Power analysis for sample size calculation was performed to determine the balance between number of subjects, size of observed effect and required alpha value. Data of FST from experiments 1 and 4 and of OFT from Experiment 4 were analyzed by independent Student's *t*-test, while data of OFT from experiments 2 and 3 were analyzed by two-way ANOVA followed by Duncan's test when appropriate. All of the data were expressed as means ± SEM. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. The results from Experiment 1 are shown in Fig. 1: influence of prolonged dietary trans fat consumption on the forced swimming task (FST)

Independent Student's *t*-test showed a significant influence of *trans* fat on the immobility and climbing/struggling time in the FST. In fact, immobility time was significantly shorter in HVF than in C-SO group ($P < 0.05$). Additionally, there was a significant difference in active behavior: climbing/struggling time was longer in HVF group ($P < 0.001$), while no significant difference in swimming time was observed.

3.2. The results from Experiment 2 are shown in Fig. 2: Influence of dietary trans fat consumption on locomotor activity in open field task (OFT)

Duncan's test showed a significant main effect of dietary consumption ($F(1,28) = 14.94$; $P < 0.000$), as well as of AMPH administration ($F(1,28) = 72.53$, $P < 0.000$). Post-hoc comparisons indicated that *trans* fat fed rats of both groups (vehicle- and AMPH-injections) showed increased locomotor activity in comparison to controls (C-SO group); However, AMPH treatment increased locomotor activity in both C-SO- and HVF- supplemented groups.

3.3. The results from Experiment 3 are shown in Fig. 3: Influence of perinatal supplementation of trans fat on locomotor activity observed in open field task (OFT)

Post-hoc comparisons by Duncan's multiple range test showed a significant influence of *trans* fat supplementation on locomotor activity observed in the open-field task: crossing number was significantly higher in the HVF group than in the control diet (C-SO group) at both evaluation times($P < 0.05$ and $P < 0.001$, respectively).

3.4. The results from Experiment 4 are shown in Figs. 4 and 5: Influence of trans fat supplementation for two generations in forced swimming test (FST) and open-field task (OFT)

Independent Student's *t*-test showed a significant influence of *trans* fat supplementation on the immobility, climbing/struggling and swimming times in FST. The immobility time was shorter in the HVF group than in the control diet (C-SO group) ($P < 0.05$). In addition, there was a significant difference in active behavior: longer climbing time and shorter swimming time in the HVF group as compared to the C-SO group ($P < 0.05$ and $P < 0.001$, respectively). As shown in Fig. 5, the HVF group displayed increased crossing and rearing behavior after AS exposure ($P < 0.05$ for both) in relation to the C-SO group.

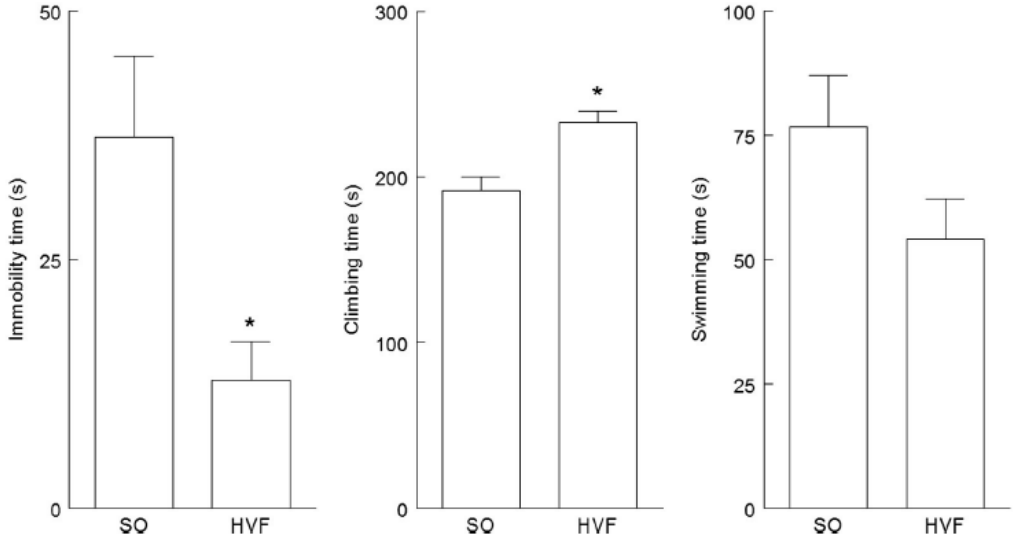

Fig. 1. Influence of diets enriched with 20% of control-soybean oil (C-SO) or hydrogenated vegetal fat (HVF) for 10 months on forced swimming task: immobility, climbing/struggling and swimming times. Data are expressed as mean ± S.E.M. * indicates significant difference between the different diets ($P < 0.05$).

4. Discussion

The different experimental trials reported here were carried out separately, as part of different studies with different aims, but the similarity of findings could not be ignored. To the best of our knowledge, this is the first experimental report of a clear connection between chronic consumption of *trans* fat and development of hyperactivity-like symptoms. Our findings showed that prolonged intake of *trans* fat in general is able to affect behavioral responses in rats. More specifically, our research showed that *trans* fat feeding for 10 months after weaning (Experiment 1), as well as during pregnancy and lactation across two generations (Experiment 4), was able to induce active coping in the FST, as judged by the reduced immobility displayed by this experimental group. Similarly, a previous study in rats reported that a high fat diet given from weaning to puberty increased active behaviors in the FST [54]. In addition, it was also demonstrated that n − 3 fatty acid deficiency diet is associated with abnormal elevations in climbing behavior in the FST following chronic fluoxetine treatment, and this response was associated with alteration in receptors expression [55]. Based on this, we suggest that regular consumption of *trans* fat during development and across two generations may have exacerbated the agitation symptoms observed in the FST. This task has been widely used to study drugs with antidepressant potential, especially because most antidepressant compounds are able to reverse FST-induced manifestations, such as increased immobility time and decreased swimming/climbing [56–58]. In fact, the presence of these behaviors in the FST has been considered as an indicator of despair behavior [50,59,60]. Contrary to this interpretation, we do not believe that the lower immobility time displayed by *trans* fat fed rats, as observed in this study, could indicate "anti-depressive behavior", but rather regard the abnormal behavior in the forced swimming task as a misreading of the situation. Alternatively, it has been proposed that increased immobility in the FST can be interpreted as a coping strategy due to a learning process based on the animal's previous experience in the same environment [54]. Detailed behavioral studies have indicated that immobility during the FST is not a failure of coping, but instead reflects a relatively successful coping strategy that employs energy conserving behaviors [61,62]. From this perspective, our findings suggest that the C-SO group used a different strategy

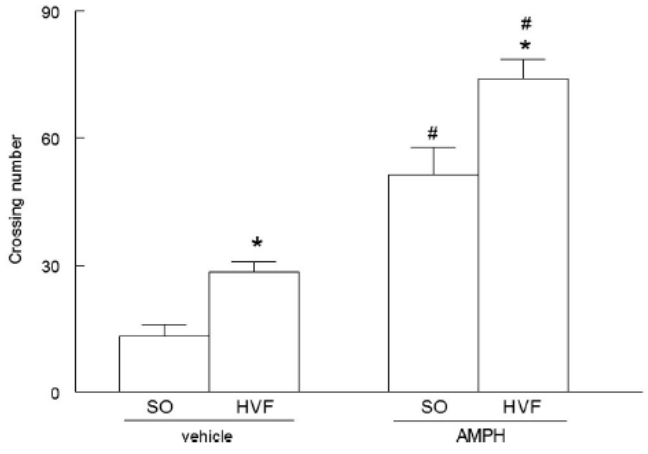

Fig. 2. Influence of dietary control-soybean oil (C-SO) or hydrogenated vegetable fat (HVF) consumption for 8 weeks on spontaneous locomotor activity in open-field task (number of crossing responses). Behavioral evaluation was carried out 2 h after the last injection of seven daily injections of dl-amphetamine (4 mg/kg/mL-ip). Data are expressed as mean ± S.E.M. * indicates significant difference between the different diets ($P < 0.05$). # indicates significant difference from vehicle within the same diet ($P < 0.001$).

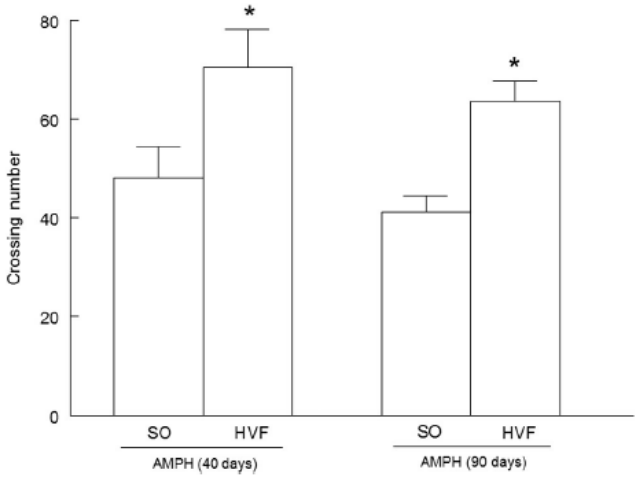

Fig. 3. Influence of supplementations with control-soybean oil (C-SO) or hydrogenated vegetable fat (HVF) on locomotor status of young and adult rats in open field task. Animals were born of dams supplemented with the same fat from gestation/lactation, and maintained in the same supplementation from weaning until 40 or 90 days of age and treated with AMPH (4 mg/kg for 8 days) until 2 h before the behavioral assessments. Data are expressed as mean ± S.E.M. * indicates significant difference between the different supplementations ($P < 0.05$).

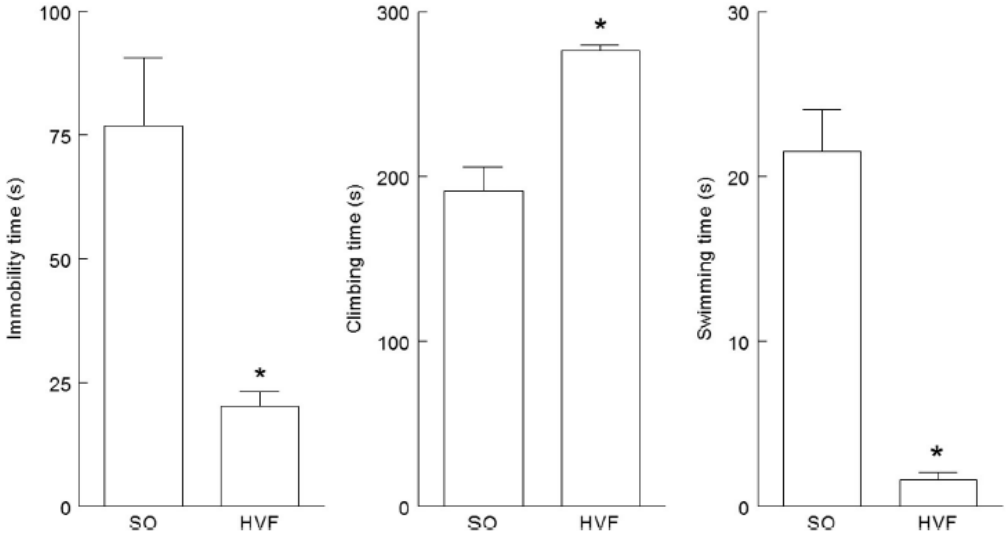

Fig. 4. Influence of supplementation with soybean oil (C-SO) or hydrogenated vegetable fat (HVF) in a second generation of young rats on forced swimming task: immobility, climbing/struggling and swimming times. Data are expressed as mean ± S.E.M. * indicates significant difference between the different supplementations ($P < 0.05$).

in order to succeed in this inescapable situation, as compared to *trans* fat fed-animals, which showed desperate and hyperactive behavior as a response to environmental stress.

In addition, human hyperactivity has been described as an inability to remain seated or keep quiet, while in animal models such behavior has been quantified by locomotor activity in the open field task (OFT). In the present study, *trans* fat consumption was associated with increased locomotion before and after administration of amphetamine (Experiment 2). Similarly, HVF supplementation during pregnancy and lactation caused increased locomotion at both evaluation times: young and adult age (Experiment 3). Similarly, *trans* fat consumption across two generations increased both locomotor and exploratory activities after exposure to stressors (Experiment 4), reinforcing our hypothesis of higher impulsiveness and reactivity to novelty in *trans* fat-fed rats in comparison to controls.

The precise pathophysiology underlying hyperactive symptoms observed in the different experimental trials performed here are currently unknown, but their occurrence may be likened to symptoms of Attention Deficit Hyperactivity Disorder (ADHD), a human condition with similarities. Interestingly, a potential involvement of dopamine receptor genes was described [63], and some studies showed a relationship between dopaminergic neurotransmission and increased locomotor activity, such as: i) *trans* fat consumption may impair the fluidity and synaptic plasticity of neural membranes [64], thus affecting dopaminergic neurotransmission [30]; ii) changes in dopamine neurotransmission are related to increased locomotor activity and hyperactivity signals; iii) human studies have reported that PUFA deficiency predisposes children to ADHD, who show behavioral symptoms of hyperactivity, delinquency, and aggression [65–67]; iv) lower brain DHA content in rats was related to more prolonged reaction to novelty [35]. Additionally, it is possible that the behavioral changes observed in this study could also involve epigenetic modifications, once HVF intake during the perinatal period could also be reflected on gene expression programming, affecting behaviors in adulthood [68].

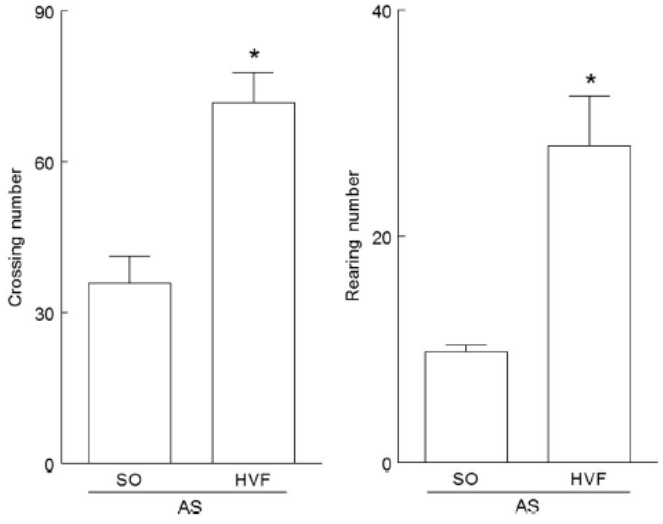

Fig. 5. Influence of supplementation with soybean oil (C-SO) or hydrogenated vegetable fat (HVF) in a second generation of young rats acutely exposed to stress on locomotor and exploratory activities in open field task. Data are expressed as mean ± S.E.M. * indicates significant difference between the different supplementations ($P < 0.05$).

We also suggest that long-term consumption of *trans* fat may have increased TFA incorporation in brain membrane phospholipids, modifying membrane fluidity and neurotransmission, as reported by Acar et al. [30]. Additionally, TFA are able to inhibit desaturases and elongases [27,69], thus hindering LC-PUFA incorporation in brain, which is crucial for normal functionality of neural membranes [7,8]. Consistent with our hypothesis, Howard et al. [70] reported a relationship between western diet, rich in *trans* fat, and ADHD diagnosis in comparison to n − 3 fatty acids-containing diets. Indeed, this idea is supported by previous studies of our group, where prolonged *trans* fat intake was related to a small but significant TFA incorporation without n − 3 FA deprivation in the brain membranes [21,25,37,71], which were related to different neuropsychiatric conditions, such as movement disorders, anxiety-like symptoms, AMPH-induced mania-like behavior, and drug addiction, respectively.

In conclusion, *trans* fat consumption from weaning until adulthood and across two generations was found to be associated with increased locomotor activity, impulsiveness and agitation behavior. These data are all the more valuable given the current scarcity of research into the effect of TFA-rich diets on behavioral problems. Our findings open up an exciting perspective for understanding the role of *trans* fat consumption on hyperactivity-like symptoms, which may indicate a predictability to ADHD development. Based on these preliminary findings, further studies are nepyeded to confirm the relationships described here, investigating its causality as well, in order to increase our

knowledge about the effects of Western diets and advise public health authorities accordingly.

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4.3 Manuscrito

MATERNAL *TRANS* FAT INTAKE DURING PREGNANCY OR LACTATION IMPAIRS MEMORY AND ALTERS BDNF AND TRKB LEVELS IN THE HIPPOCAMPUS OF ADULT OFFSPRING EXPOSED TO CHRONIC MILD STRESS

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Status: **Em fase de submissão**

Maternal *trans* fat intake during pregnancy or lactation impairs memory and alters BDNF and TrkB levels in the hippocampus of adult offspring exposed to chronic mild stress.

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Abstract

Maternal nutrition during the first stages of life is directly related to the adequate development of the fetus, the newborn, and the future adult. This study aimed to assess the influence of maternal dietary fat intake during pregnancy or lactation on memory of adult offspring after chronic mild stress exposure. Female Wistar rats were supplemented daily with soybean oil/fish oil (SO/FO, optimal ratio of n-6/n-3 fatty acids) or hydrogenated vegetable fat (HVF, rich in *trans* fatty acids - TFA) by oral gavage (3.0 g/kg body weight) during pregnancy or lactation. After weaning, the male offspring from both experimental groups received only standard diet. On post-natal day (PND) 60, half of the animals of each experimental group were exposed to the chronic mild stress (CMS) procedure following behavioral assessments. While the adult offspring born under influence of SO/FO and HVF supplementations during pregnancy showed higher levels of n-3 and n-6 fatty acids (FA) series DHA (22:6 n-3) and ARA (20:4 n-6) metabolites, respectively, in the hippocampus, adult offspring born from SO/FO- and HVF-supplemented dams during lactation showed higher levels of their precursors: ALA (18:3 n-3) and LA (18:2 n-6), respectively, in the same brain area. However, only HVF supplementation allowed TFA incorporation in the hippocampus of adult offspring, and levels were higher in animals exposed to this FA during lactation in comparison to those exposed during pregnancy. Adult offspring born from dams supplemented with *trans* fat in both pregnancy and lactation showed short and long-term memory impairments before and after CMS exposure, being that the stress protocol was able to increase short-term memory in SO/FO and decrease both short and long-term memory in animals born from HVF-supplemented dams during pregnancy. Furthermore, our study also showed higher memory impairment in offspring born from HVF-supplemented dams during lactation in comparison to pregnancy, regardless of stress exposure. BDNF expression was increased by stress exposure in offspring born from both SO/FO- and HVF-supplemented dams during pregnancy, but not when these fats were supplemented during lactation. In addition, offspring from HVF-supplemented dams showed decreased TrkB expression in both supplemented periods, regardless of stress exposure. In conclusion, these findings show for the first time that the type of dietary FA as well as the period of brain development are able to change FA incorporation in brain neural membranes, thus modifying the expression of BDNF and its receptor TrkB, which may be reflected on the cognitive function of adult offspring.

Keywords: *trans* fatty acids; early developmental period; stress exposure; memory; neurotrophin; adult offspring.

1. Introduction

In recent decades, eating habits have changed dramatically. The food industry has greatly increased the production of food with high levels of *trans* fatty acids (TFA), which are unsaturated fatty acids with at least one double bond in the *trans* molecular configuration formed during partial hydrogenation of vegetable oils (Sun et al., 2007; Mozaffarian et al., 2006), and whose increased consumption may represent about 1.7% and 8% of the world dietary fat intake by most people depending on the country (Osso et al., 2008; Eckel et al., 2007; Henderson et al., 2003). Different studies have shown an interesting relationship between dietary *trans* fat intake and risk of inflammation (Mozaffarian et al., 2004), cardiovascular disease (Sun et al., 2007; Hu et al., 1997) and cognitive dysfunction (Fillit et al., 2008; Kodl and Seaquist 2008; Morgan et al., 2007; Yaffe 2007; Razay et al., 2006). Moreover, TFA may be easily incorporated into neural membrane phospholipids, altering membrane fluidity, biochemical properties, and affecting cell function (Larqué et al., 2003; Grandgirard et al 1998; Morgado et al., 1998).

In this sense, the influence of dietary lipid type in early stages of life on the health of adult offspring is of great importance, especially because its incorporation into neural membranes occurs at a higher intensity during the developmental period, affecting synaptic plasticity and neural reorganization (de Velasco et al., 2012; Ibrahim et al., 2009; Khan et al., 2005). Inadequate maternal nutrition during neonatal period can change both morphological and physiological parameters of pups, since maternal intake of FA determines the transfer of essential fatty acids (EFA) or non-essential TFA through the placenta, milk and in a later stage through dietary sources (Innis, 2007). Although there was a limited transfer of fatty acids by the placenta, it has been demonstrated that changes in dietary FA have implications in the development of fetal and postnatal central nervous systems (CNS)(Herrera 2002), since they are important constituents of cell membranes, where they exert a fundamental role on cortical maturation, synaptogenesis and myelination (Hanebutt et al., 2008). Different studies from our laboratory have shown that *trans* fat intake of female rats during pregnancy and lactation was related to increased oxidative damage in mesocorticolimbic brain areas of their offspring (Trevizol et al., 2013; 2011). Also, intake of hydrogenated vegetable fat, which is rich in TFA, over one or two generations of rats, was related to addiction-related factors (Kuhn et al., 2015; 2011), anxiety-like symptoms (Pase et al., 2013), hyperactive behavior (Pase et al., 2015) and development of bipolar disorder, followed by higher incorporation of *trans* fatty acids in different brain areas (Trevizol et al., 2015; 2014). Conversely, fish oil supplementation, rich in

n-3 FA, was able to exert an evident protective role, reducing oxidative damages in different brain areas related to neuropsychiatric diseases (Trevizol et al., 2013; 2011). In fact, these findings indicate that FA composition of maternal diet during pregnancy and/or lactation is a critical factor strongly associated with normal fetal and postnatal development, and also seem to predispose the offspring to behavioral disorders throughout life.

On the other hand, stress has also been associated with the development of neuropsychiatric conditions, which are able to exert significant influence on development and course of CNS diseases (Karatsoreos and McEwen, 2011; Sapolsky et al., 2000). Stress alters neurotransmission and synaptic plasticity in brain areas involved in the limbic–hypothalamic–pituitary–adrenal axis (Gardner et al., 2009; Dunn and Swiergiel, 2008). Long lasting and intensive stimuli can lead to a persistent change in stress response and mechanisms, and in function and structure of the brain itself (Armario et al., 2008; Darnaudery and Maccari, 2008). The hippocampus is particularly susceptible to chronic stress-induced neuronal damage, including dendritic atrophy, decreased neurogenesis and synaptic plasticity, which results in impaired cognitive function (Zafir and Banu, 2009) and behavior alterations (Winocur et al., 2012; Wellman et al., 2011; Raju et al., 2007) that can translate into neurodegenerative or mental illnesses (Corcoran et al., 2003). Recent evidences have indicated that the neurotrophin brain-derived neurotrophic factor (BDNF) is considered an important factor to regulate synaptogenesis and synaptic plasticity, which are associated to learning and memory functions (Gomez-Palacio-Schjetnan and Escobar, 2013; Cunha et al., 2010). Thus, BDNF may be considered a molecular target for mediation of negative impact of stress events on brain structure and function (Cirulli et al., 2009).

Considering that the type of dietary fatty acids received during early life periods can make the CNS more vulnerable to stress, and more susceptible to the development of neuropsychiatric diseases, the aim of this study was to investigate the influence of maternal *trans* fat intake during pregnancy and lactation on fatty acids profile in the hippocampus of adult male offspring. In addition, the influence of *trans* fat intake during the perinatal period together with chronic stress exposure on memory impairments and brain levels of BDNF of the same male adult offspring was assessed, since evidence suggests that changes in BDNF levels might play a role in mediating cognitive function,

2. Materials and methods

2.1 Animals

All animal procedures were approved by the Research Ethics Committee of the Universidade Federal de Santa Maria. Animals were maintained and used in accordance with the guidelines of the Brazilian Association for Laboratory Animal Science (COBEA). They were kept in Plexiglas cages with free access to food and water in a room with controlled temperature (23°C±1) on a 12 h-light/dark cycle throughout the experimental period. Pregnant female Wistar rats (n = 40) were randomly assigned to two experimental groups (n=20 for each group): soybean oil/fish oil group (SO/FO, optimal ratio of n-6/n-3 fatty acids) or hydrogenated vegetable fat group (HVF, rich in *trans* fatty acids) (Table 1), which were supplemented daily by oral gavage with 3.0 g/kg body weight (Ferraz et al., 2008) during pregnancy (n=10) or lactation (n=10). After weaning, the male offspring from all groups received only standard diet. On post-natal day 60, half of the adult male offspring of each supplemented group (n=5) were exposed to the chronic mild stress (CMS) procedure described as follows. After 24 h of CMS exposure, animals were submitted to behavioral assessments. Diets were isocaloric and normolipidic, differing only in the fat source. SO and HVF were purchased in a local supermarket and FO was donated by Herbarium® (Curitiba, Brazil).

2.2 Chronic mild stress (CMS)

A modified version of the CMS protocol first described by Willner et al. (1987) was used. Rats were daily exposed to different tips of stressors following a semi-randomized schedule that included damp sawdust, grouped housing, cage tilting (45°), lights on overnight, isolation, switching cages, and foreign object in cage for 3 weeks. The stress procedure did not include any food or water deprivation (Kompagne et al. 2008). Rats received one of these stressors per day in the sequence shown above and the same stressor was not applied in two subsequent days. The stress procedure lasted 3 weeks prior to behavioral testing.

2.3 Behavioral paradigms

2.3.1 Novel object recognition task (NORT)

This paradigm is related to animals' natural motivation to explore novelties considered an innate instinct they use to recognize their environment (Heldt et al., 2007), and a higher score implies a higher recognition rate, indicating better memory. Recognition memory was assessed as previously described (De Lima et al., 2005): the arena floor was covered with sawdust (from bedding material) during recognition memory training and test trials. On the first day, rats were given one training trial being exposed to two identical objects (A1 and A2, double Lego toys) positioned in two adjacent corners and they were allowed to freely explore the objects for 5 min

(training session). Testing of short-term memory (STM) and long-term memory (LTM) was performed 1 and 24 hours after the training session, respectively. Rats were allowed to explore the open field for 5 min in the presence of two objects: the familiar Object A and a second novel Object B or C, which were placed in the same locations of the training session. All objects had similar textures, colors, and sizes, but distinctive shapes. Objects were cleaned between trials with a 5% alcohol solution; exploration was defined as sniffing or touching the object with the nose. A recognition index calculated for each animal was expressed by the ratio TN/(TF+TN) (TF= time spent exploring the familiar object; TN= time spent exploring the novel object).

2.4 Tissue Preparations

Following (24 h) the last behavioral assessments, all animals were anesthetized (sodium pentobarbital, 50 mg/Kg body weight ip) and euthanized by exsanguinations. The collected blood (collected by cardiac puncture in heparinized tubes) was centrifuged at 3,000g (15 min) for plasma and used for biochemical assay. Brains were removed and cut coronally at the caudal border of the olfactory tubercle to remove the hippocampus (Paxinos and Watson, 2007). The tissue was separated into two parts, one of which was used to determine fatty acids profile, and the second part was stored in a freezer at -80° C for subsequent molecular analysis.

2.5 Fatty acid (FA) profile in brain tissue

Fat was extracted from the hippocampus samples using chloroform and methanol as described by Bligh and Dyer, 1959, and used for determination of FA profile. To prevent lipid oxidation during and after extraction, 0.02% butyl hydroxy toluene was added to the chloroform used. FA composition was determined by gas chromatography. Fat was saponified in methanolic KOH solution and then esterified in methanolic H_2SO_4 solution (Hartmann and Lago, 1973). Methylated fatty acids were analyzed using a gas chromatograph (Agilent Technologies, HP 6890N) equipped with a capillary column DB-23 (60 m x 0.25 mm x 0.25 mm) and a flame ionization detector. The temperature of the injector port was set at 280°C and the carrier gas was nitrogen (0.9 mL/min). After injection (1 µL, split ratio 50:1), the oven temperature was held at 160°C for 1 min, then it was increased to 240°C at 4°C/min and held at this temperature for 9 min. Standard FA methyl esters (37-component FAME Mix, C 22:5n3 and PUFA no. 2 from Sigma, Saint Louis, MO and C 22:5n-6 from NuChek Prep. Inc., Elysian, MN) were run under the same conditions and the subsequent retention times were used to identify the FA. FA was expressed as percentage of the total FA content.

2.6 Corticosterone assay

Quantification of corticosterone levels was assessed in the serum samples by ELISA using commercial kits (Abcam Products, San Francisco, CA, USA), according to the manufacturer's instructions.

2.7 Molecular assessments

Immunoblotting analysis of brain derived neural factor (BDNF) and tropomyosin-related kinase B (TrkB) density:

The hippocampal tissue was homogenized in a lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP40, 10% glycerol, 1 mM PMSF, 10 ug/ml-1 aprotinin, 0.1 mM benzethonium chloride, 0.5mM sodium vanadate. Homogenates were then centrifuged, supernatants collected and total protein concentration was determined according to MicroBCA procedure (Pierce, IL, USA), using bovine serum albumin as standard. Levels of brain derived neurotrophic factor (BDNF), tropomyosin-related kinase B (TrkB) and actin were analyzed by Western blot. Briefly, protein samples were separated by electrophoresis on a 10% polyacrylamide gel and electrotransferred to a PVDF membrane (Millipore, MA, USA). Non-specific binding sites were blocked in Tris-buffered saline (TBS) overnight, at 4 °C, with 2% BSA and 0.1% Tween-20. Membranes were rinsed in buffer (0.05% Tween-20 in TBS) and incubated with primary antibodies: anti-actin (1:1000; Santa Cruz Biotechnology, CA, USA), anti-BDNF (1:500; Santa Cruz Biotechnology, CA, USA), and anti-TrkB (1:1000; Santa Cruz Biotechnology, CA, USA), followed by anti-goat (1:25.000; Santa Cruz Biotechnology, CA, USA) and anti-rabbit (1:8000; Santa Cruz Biotechnology, CA, USA) IgG horseradish peroxidase conjugate, respectively. After rinsing with buffer, immunocomplexes were visualized using 3,3',5,5'– Tetramethylbenzidine (TMB) (Sigma, USA) according to the manufacturer's instructions. Film signals were digitally scanned and then quantified using ImageJ software. Actin was used as an internal control for western blot so that data were standardized according to actin values.

2.8 Statistical analysis

FA content in the different supplementations (SO/FO and HVF) were analyzed by one-way ANOVA, while their incorporation in the hippocampus was analyzed by two-way ANOVA (2 supplementations (SO/FO and HVF) × 2 periods (pregnancy and lactation)). Behavioral, biochemical and molecular assessments were analyzed by three-way ANOVA (2 supplementations (SO/FO and HVF) × 2 periods (pregnancy and lactation) x 2 stress exposure

(NS and CMS)). All statistical tests (ANOVA) were followed by Duncan's multiple range test, when appropriate. $P<0.05$ was regarded as statistically significant.

3. Results

3.1 Time spent on the Novel object recognition task (NORT) is shown in Figure 1 and Figure 2.

Three-way ANOVA revealed a significant main effect of supplementation [$F(1,32) = 44.06$, $P<0.001$] on time spent in the NORT after 1 h, and a significant main effect of supplementation, period and supplementation × period interaction [$F(1,32) = 157.47$, $P<0.001$; $F(1,32) = 29.38$, $P<0.001$; $F(1,32) = 7.44$, $P<0.05$], respectively, on time spent in the NORT after 24 h.

Post-hoc analysis showed that HVF supplementation during pregnancy and lactation decreased time spent in the NORT in both short (1 h) and long-term (24 h) memory in animals exposed or not exposed to the stress protocol. After 1 h, CMS exposure showed significant influence only on offspring supplemented during pregnancy, where rats from SO/FO supplementation exposed to CMS showed increased time spent in the NORT, whereas this behavior was decreased in HVF group (Fig. 1A). The CMS protocol also decreased long-term memory, which was observed by less time spent in the NORT after 24 h in HVF group (Fig. 1B). Exposure to CMS had no significant differences on offspring supplemented during lactation. Between the different periods of supplementation, offspring from HVF-supplemented dams during lactation showed lower recognition index (24 h) than the pregnancy period (Fig. 1B).

3.2 Fatty acids composition quantified in the hippocampus of adult offspring is shown in Table 2.

Two-way ANOVA revealed a significant main effect of supplementation, period and supplementation × period interaction [$F(1,32) = 8.93$, $P<0.001$; $F(1,32) = 43.32$, $P<0.001$; $F(1,32) = 18.48$, $P<0.001$, respectively] on total MUFA incorporation; a significant main effect of period and supplementation × period interaction [$F(1,32) = 22.73$, $P<0.001$; $F(1,32) = 2.87$, $P<0.05$, respectively] on total PUFA incorporation; a significant main effect of supplementation and period [$F(1,32) = 4.30$, $P<0.05$; $F(1,32) = 121.4$, $P<0.001$, respectively] on LA incorporation; a significant main effect of period and supplementation × period interaction [$F(1,32) = 50.06$, $P<0.001$; $F(1,32) = 3.01$, $P<0.05$, respectively] on ARA incorporation; a

significant main effect of supplementation, period and supplementation × period interaction [F(1,32) = 11.89, *P*<0.001; F(1,32) = 58.15, *P*<0.001; F(1,32) = 13.84, *P*<0.001, respectively] on ALA incorporation; a significant main effect of supplementation, period and supplementation × period interaction [F(1,32) = 13.06, *P*<0.001; F(1,32) = 62.56, *P*<0.001; F(1,32) = 8.28, *P*<0.001, respectively] on DHA incorporation; a significant main effect of supplementation, period and supplementation × period interaction [F(1,32) = 43.02, *P*<0.001; F(1,32) = 63.11, *P*<0.001; F(1,32) = 8.28, *P*<0.001, respectively] on DHA incorporation; a significant main effect of supplementation, period and supplementation × period interaction [F(1,32) = 43.02, *P*<0.001; F(1,32) = 63.11, *P*<0.001; F(1,32) = 4.14, *P*<0.001, respectively] on n-3 PUFA incorporation; a significant main effect of period [F(1,32) = 232.48, *P*<0.001] on n-6 PUFA incorporation; a significant main effect of supplementation, period and supplementation × period interaction [F(1,32) = 261.6, *P*<0.001; F(1,32) = 10612.6, *P*<0.001; F(1,32) = 67.1, *P*<0.001, respectively] on n-6/n-3 FA ratio incorporation and a significant main effect of supplementation and supplementation × period interaction [F(1,32) = 36.50, *P*<0.001; F(1,32) = 10.23, P<0.001, respectively] on TFA incorporation.

Duncan's test showed that HVF supplementation during pregnancy was able to increase ∑MUFA by 7.99% and decrease DHA (22:6 n-3) by 9.14%, ∑PUFA by 4.18% and ∑n-3 PUFA by 9.33% in the hippocampus of adult offspring in comparison to SO/FO group. In contrast, during lactation, HVF supplementation decreased LA (18:2 n-6) by 14.42%, ALA (18:3 n-3) by 85.18% and ∑n-3 PUFA by 9.10%. In addition, only HVF supplementation in both perinatal periods was related to a significant TFA incorporation in the hippocampus of offspring, which was lower when HVF supplementation was provided during pregnancy (0.18%) in comparison to lactation (0.43%). Post-hoc analysis also showed that adult offspring born from both SO/FO and HVF- supplemented dams during lactation showed lower incorporation of ARA (20:4 n6), DHA (22:6 n3), ∑PUFA, and ∑n-3 PUFA and higher incorporation of LA (18:2 n6) in relation to pregnancy (*P*<0.05 for all comparisons).

3.2 Corticosterone levels in plasma of adult offspring is shown in Figure 2.

Three-way ANOVA revealed a significant main effect of supplementation, period, and stress protocol [F(1,32) = 191.60, *P*<0.001; F(1,32) = 60.11, *P*<0.001; F(1,32) = 187.54, *P*<0.001] on corticosterone (CORT) levels.

Post-hoc test showed that offspring born from HVF-supplemented mothers in both pregnancy and lactation showed increased plasma levels of CORT in relation to SO/FO supplementation, regardless of the stress condition. CMS exposition increased CORT levels in

offspring born from both SO/FO and HVF-supplemented mothers, whose value was higher in lactation compared to pregnancy.

3.3 BDNF and TrkB levels quantified in the hippocampus of adult offspring are shown in Figure 3 and 4.

Three-way ANOVA revealed a significant main effect of period and stress protocol [$F(1,32) = 203.31$, $P<0.001$; $F(1,32) = 194.92$, $P<0.001$] and supplementation × period, supplementation × stress protocol, period × stress protocol and supplementation × period × stress protocol interaction [$F(1,32) = 3.92$, $P<0.05$; $F(1,32) = 5.09$, $P<0.05$; $F(1,32) = 211.24$, $P<0.001$, $F(1,32) = 4.75$, $P<0.05$ respectively] on BDNF levels in hippocampus. Three-way ANOVA revealed a significant main effect of supplementation, period, stress protocol and period × stress protocol interaction [$F(1,32) = 33.97$, $P<0.001$; $F(1,32) = 12.49$, $P<0.001$; $F(1,32) = 21.21$, $P<0.001$; $F(1,32) = 10.17$, $P<0.05$, respectively] on TrkB levels in the hippocampus of adult offspring.

Post-hoc test showed that HVF supplementation during pregnancy and lactation exerted no influence on BDNF levels in the hippocampus of offspring. CMS exposure increased BDNF levels in the hippocampus of offspring born from both SO/FO and HVF-supplemented dams during pregnancy only, and levels were higher in HVF group. Considering differences between the two periods of supplementation, BDNF was higher in offspring exposed to CMS, which were born from SO/FO and HVF-supplemented dams during pregnancy, in comparison to lactation.

Post-hoc test showed that offspring born from HVF-supplemented dams during pregnancy and lactation showed decreased TrkB levels, regardless of the stress condition. In addition, CMS exposure was able to decrease TrkB levels only in offspring born from HVF-supplemented dams during lactation in relation to SO/FO group, whose value was also lower than that observed in offspring born from HVF-supplemented dams during pregnancy.

4. Discussion

In the present study we assessed the influence of HVF supplementation during either pregnancy or lactation of female rats on FA profile in the brain of adult offspring, as well as whether this incorporation in the hippocampus could exert significant influence on the memory acquisition process, which is closely related to molecular targets (BDNF and TrkB) in this brain

area. Following the study, the same behavioral and molecular parameters were assessed after exposure of the offspring to a protocol of chronic mild stress (CMS), in adulthood.

In fact, FA accumulation in fetal and infant tissues, particularly the brain, can be influenced by the pre and post-natal FA supply (Innis, 2011). Thus, in early stages of life, the offspring is completely dependent on the maternal intake of FA (Brenna and Carlson, 2014; Innis, 2007), suggesting that imbalance of maternal dietary fat composition can impair the brain development of offspring since these FA have the ability to pass through the blood brain barrier (Bolton and Bilbo, 2014). Some authors have shown that the placental barrier present binding sites of high selectivity to transfer long chain polyunsaturated fatty acids (LC-PUFA), such as DHA and ARA (Duttaroy, 2009; Hanebutt et al., 2008; Herrera et al., 2006). In contrast, Campbell et al. (1996) have shown that TFA are able to compete for the same LC-PUFA binding sites in human placental membranes, inhibiting the transport of LC-PUFA through the placenta (Campbell et al., 1996). Of particular interest for our findings, a negative association between TFA and n-3 LC-PUFA in fetal plasma was recently observed (Enke et al., 2011), and a decrease in the total levels of PUFA, ARA and DHA in the brain of offspring born from mothers fed with a TFA-enriched diet during the perinatal period (Albuquerque et al., 2006). According to these studies, our current findings showed that only HVF intake was related to incorporation of TFA in the hippocampus of adult offspring, however, this incorporation was favored when HVF was provided during lactation, since these offspring showed higher levels of TFA in the hippocampus than those animals born from mothers supplemented with this fat during pregnancy. Moreover, *trans* fat intake during pregnancy was associated with decreased levels of DHA, n-3 PUFA and total PUFA, while *trans* fat intake during lactation was related to decreased LA, ALA and n-3 PUFA incorporation. Comparisons between supplementation periods revealed that both supplementations SO/FO and HVF were able to decrease incorporation of n-3 and n-6 PUFA metabolites (DHA-22:6 n-3 and ARA-20:4 n-6, respectively) and increase incorporation of LA (18:2 n-6), which is a precursor of ARA, during lactation in comparison to pregnancy. Additional comparisons between the two different periods of supplementation with HVF, which is rich in TFA, allow us to show for the first time that the ingestion of this fat during lactation was able to significantly modify incorporation of FA in the hippocampus, in relation to pregnancy, as follows: i) increased TFA, as described above, and increased SFA level; ii) decreased n-3 PUFA, PUFA and MUFA.

Based on these outcomes, we hypothesized that the different incorporation of FA in the hippocampus during pregnancy and lactation may be due to the presence of binding sites, which facilitate the passage of LC-PUFA through the placenta in detriment of TFA, reinforcing the

hypothesis that LC-PUFA are more easily incorporated into neuronal membranes during the period of cerebral development (Bourre et al., 1989). In fact, the placenta barrier presents high selectivity for LC-PUFA, since these FA are nutrients required for synthesis of structural lipids, which are fundamental to fetal and postnatal development and normal cell function (Herrera et al., 2006; 2002; Innis 2005). For the first time these findings allow us to hypothesize that not only the type of FA but also the period of development of its provision may be determinant for brain neural membranes FA composition, whose imbalance or food deficiency may produce adverse consequences for the fetus, the newborn, and the adult offspring (Herrera 2002).

Our earlier study demonstrated increased susceptibility of animals born and/or grown under the influence of *trans* fat to develop memory impairment, movement disorders and hyperlocomotion related to manic behavior (Trevizol et al., 2013, 2011; Teixeira et al., 2012, 2011). *Trans* fat intake during early periods of life was also related to increased preference for psychostimulant drugs (Kuhn et al., 2013), facilitating the development of anxiety-like symptoms in young rats born from second generation (Pase et al., 2013). In the current study, we observed that adult offspring born from *trans* fat-supplemented dams during both pregnancy and lactation showed impairments in short and long-term memory, regardless of chronic mild stress (CMS) exposure. In addition, CMS exposure was able to increase short-term memory in offspring born from SO/FO supplemented dams and decreased both short and long-term memory in animals born from HVF-supplemented dams during pregnancy. Interestingly, our outcomes showed higher memory impairment in offspring born from HVF-supplemented dams during lactation, regardless of CMS exposure, in comparison to pregnancy. Interestingly, studies support the involvement of both BDNF and TrkB-receptor in learning and memory (Kemppainen et al., 2012). Here, BDNF levels were increased by stress condition on both SO/FO- and HVF-supplemented groups during pregnancy and no difference was observed during lactation. In addition, only offspring from HVF-supplemented dams during both pregnancy and lactation showed decreased TrkB levels, regardless of the stress condition.

As the hippocampus is the main brain area related to spatial memory (Martin and Clark, 2007), we believe that the variation in FA composition observed in this study might be correlated with cognitive impairments in adult offspring. Some authors have shown that changes in lipid composition and TFA incorporation in neural membranes may affect synaptic plasticity (Larqué et al., 2003), modifying neurotransmission and leading to significant conformational modifications in membrane-bound proteins. In this sense, we suggest that significant *trans* FA incorporation in the hippocampus of offspring born from HVF-supplemented dams in different stages of development may be related to a decreased density of

the TrkB receptor, as observed in the current study, whose consequence was impairment in memory performance. It is especially important to note that lower levels of TrkB were only observed in adult offspring born from HVF-supplemented dams during lactation, which was corresponding to higher TFA incorporation and higher long-term memory impairment. Taken together, these findings confirm that TFA incorporation in neural membranes can modify its fluidity and change responses related to membrane receptors. These outcomes are in accordance with other authors, when impaired TrkB receptor signaling contributes to memory loss (Kemppainen et al., 2012) and TrkB null mice show a dramatic deficit in complex learning paradigms (Minichiello et al., 2009). Furthermore, while the brain up-regulation of TrkB signaling reverses cognitive deficits in an animal model of schizophrenia (Kutiyanawalla et al., 2012), mice overexpressing full-length TrkB showed improved cognitive skills (Pinilla et al., 2001).

The influence of stress on cognitive and neuronal functions have also been studied (Conrad 2010; Shors 2004), specifically because stress is able to induce alterations in gene expression for some brain proteins such as neurotrophins, in particular brain-derived neurotrophic factor (BDNF), which is related to learning and memory (Lashgari et al., 2006). Different studies have shown that exposure to acute (Lee et al., 2008) and chronic (Rothman et al., 2012; Nair et al., 2007; Tsankova et al., 2006) stressors can significantly cause a downregulation in both BDNF mRNA expression and protein levels in the hippocampus, since exposure to elevated corticosterone levels reduces BDNF expression (Rothman et al., 2012). However, Marmigere et al. (2003) found that short-term intensive stress was related to an increased BDNF gene expression, but when stress exposure was prolonged, this effect on BDNF expression was decreased (Marmigere et al., 2003), revealing that although BDNF can show significant responses to stress (Smith et al., 1995), the direction of such influences (increase or decrease) can range in different investigations (Marmigere et al., 2003; McAllister et al., 1999). In our study, adult offspring born from both SO/FO and HVF-supplemented dams during pregnancy exposed to CMS showed increased BDNF levels. Taking into account recent data from the literature, we can suggest that increased levels of BDNF after stress exposure were related to improved short-term memory in offspring born from SO/FO-supplemented dams during pregnancy, as already suggested by other authors (Liu et al., 2014; Alomari et al., 2013). In contrast, the overexpression of this neurotrophin observed in HVF group resulted in cognitive deficits of these animals. These findings are in accordance with Cunha et al. (2009), who observed learning deficits and memory impairments after chronic BDNF overexpression,

suggesting that a widespread increase of BDNF in brain networks may result in adverse effects on learning and memory formation.

In conclusion, our research show for the first time that: i) incorporation of FA in neural membranes of the hippocampus may be modified according to the period of brain development; ii) dietary *trans* FA are more easily incorporated in neural membranes of the hippocampus during lactation, in relation to pregnancy; iii) offspring born from HVF-supplemented dams during lactation presented impaired cognitive performance without influence from stress exposure in adulthood; iv) lower incorporation of TFA in the hippocampus during pregnancy was also related to memory impairment, however it was exacerbated by stress exposure, reflecting on molecular neuroadaptations. Taken together, these findings exert implications on the fat quality of foods eaten by mothers during the early periods of pups' development, whose incorporation of FA in the brain, especially the hippocampus, is able to "program" structure and memory functions throughout adult life. Starting from the premise that the human species is more sensible to damages from external influences than other species, the quality of dietary habits of pregnant and lactating women should be monitored, especially regarding the content of *trans* fat, which is often omitted or masked on labels of processed foods.

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Figure captions

Figure 1: Influence of HVF supplementation during early life periods on memory of adult offspring after chronic mild stress exposure. Animals were born from dams supplemented with different oil/fat during pregnancy or lactation. Short-term memory and long-term memory retention tests were performed 1 h (A) and 24 h (B) after training, respectively. Data are expressed as mean ± S.E.M. SO, soybean oil; FO, fish oil; HVF, hydrogenated vegetable fat. Lowercase indicates significant difference among SO/FO and HVF groups in the same stress condition (P<0.05). * indicates significant difference of stress condition in the same supplemented group (P<0.05). # indicate significant difference between the different periods (pregnancy or lactation) in the same supplemented group (P<0.05).

Figure 2: Influence of HVF supplementation during early life periods on corticosterone levels of adult offspring after chronic mild stress exposure. Animals were born from dams supplemented with different oil/fat during pregnancy or lactation. Data are expressed as mean ± S.E.M. SO, soybean oil; FO, fish oil; HVF, hydrogenated vegetable fat. Lowercase indicates significant difference among SO/FO and HVF groups in the same stress condition (P<0.05). * indicates significant difference of stress condition in the same supplemented group (P<0.05). # indicates significant difference between different period (pregnancy or lactation) in the same supplemented group (P<0.05).

Figure 3: Influence of HVF supplementation during early life periods on BDNF levels in the hippocampus of adult offspring after chronic mild stress exposure. Animals were born from dams supplemented with different oil/fat during pregnancy or lactation. Data are expressed as mean ± S.E.M. SO, soybean oil; FO, fish oil; HVF, hydrogenated vegetable fat. Lowercase indicates significant difference among SO/FO and HVF groups in the same stress condition (P<0.05). * indicates significant difference of stress condition in the same supplemented group (P<0.05). # indicates significant difference between different periods (pregnancy or lactation) in the same supplemented group (P<0.05).

Figure 4: Influence of HVF supplementation during early life periods on TrkB levels in the hippocampus of adult offspring after chronic mild stress exposure. Animals were born from dams supplemented with different oil/fat during pregnancy or lactation. Data are expressed as

mean ± S.E.M. SO, soybean oil; FO, fish oil; HVF, hydrogenated vegetable fat. Lowercase indicates significant difference among SO/FO and HVF groups in the same stress condition ($P<0.05$). * indicates significant difference of stress condition in the same supplemented group ($P<0.05$). # indicates significant difference between different periods (pregnancy or lactation) in the same supplemented group ($P<0.05$).

Table 1. Fatty acid composition (% of total identified FA) of the dietary supplementation

Fatty acids (∑)	**SO/FO**	**Hydrogenated vegetable fat**
SFA	26.91	33.58
MUFA	25.68	53.77
PUFA	47.40	12.65
n-6 PUFA	23.74	12.29
n-3 PUFA	23.58	0.36
TFA	0.16	13.43
n6/n3 ratio	1.01	34.02

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, *trans* fatty acids.

Table 2. Hippocampus fatty acids composition of adult offspring from dams supplemented with different oil/fat during gestation or lactation period (% of total fatty acids identified)

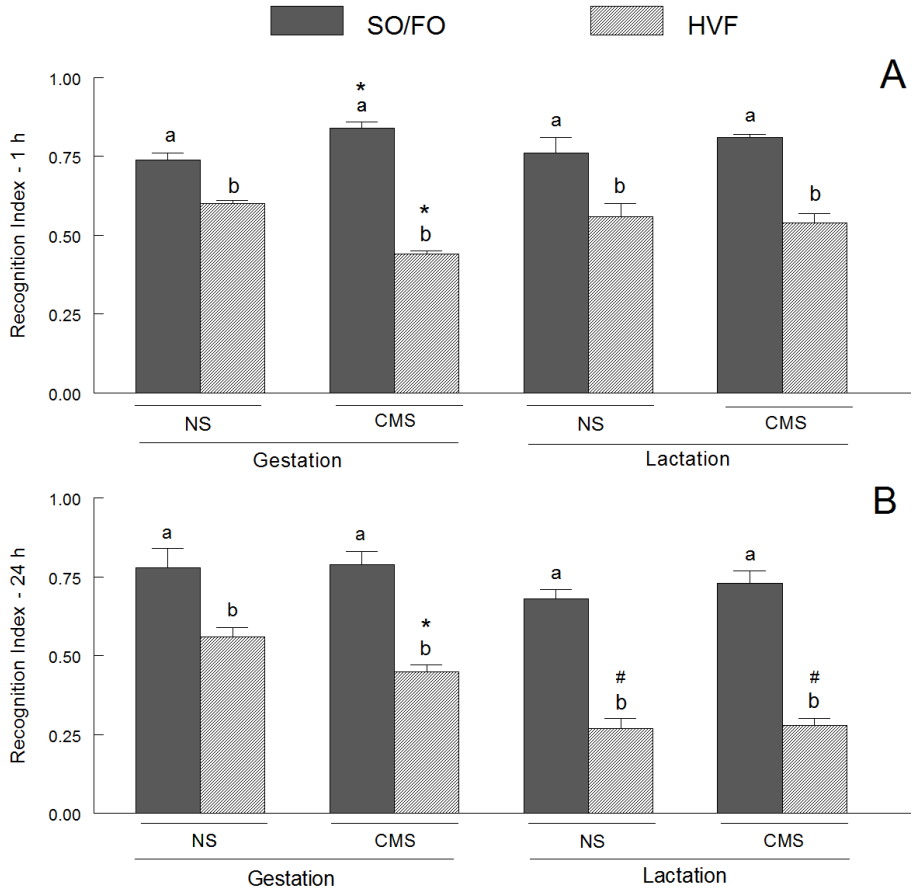
Fatty acid	Mean (±S.E.M.)			
	Gestation		Lactation	
	SO/FO	Hydrogenated vegetable fat	SO/FO	Hydrogenated vegetable fat
16:0	21.90 ± 0.31 [a]	20.90 ± 0.29 [b]	22.80 ± 0.04	22.70 ± 0.26 [#]
18:0	21.11 ± 0.03	21.22 ± 0.17	21.21 ± 0.11	21.43 ± 0.03
∑ SFA	46.62 ± 0.37	45.21 ± 0.42	47.23 ± 0.54	48.01 ± 0.03 [#]
15:1 n-5	3.15 ± 0.27 [b]	4.80 ± 0.19 [a]	2.29 ± 0.11 [#]	2.34 ± 0.04 [#]
16:1 n-7	0.65 ± 0.02	0.64 ± 0.00	0.70 ± 0.02	0.74 ± 0.03 [#]
18:1 n-7	3.26 ± 0.15	3.42 ± 0.05	2.85 ± 0.09 [#]	3.0 ± 0.03 [#]
18:1 n-9	16.78 ± 0.09	17.15 ± 0.59	17.87 ± 0.54	17.71 ± 0.38
18:1 n-9t	n.d. [b]	0.18 ± 0.00 [a]	n.d. [b]	0.43 ± 0.08 [a #]
20:1 n-9	0.85 ± 0.06	0.77 ± 0.01	0.87 ± 0.02 [b]	1.14 ± 0.13 [a #]
24:1 n-9	0.36 ± 0.02	0.31 ± 0.00	0.59 ± 0.01 [a #]	0.43 ± 0.01 [b #]
∑ MUFA	25.64 ± 0.17 [b]	27.69 ± 0.59 [a]	26.33 ± 0.23	25.90 ± 0.41 [#]
18:2 n-6	1.12 ± 0.08	1.18 ± 0.02	2.45 ± 0.23 [a #]	2.10 ± 0.07 [b #]
20:2 n-6	0.12 ± 0.01	0.10 ± 0.01	0.08 ± 0.02 [a]	0.00 ± 0.00 [b #]
20:3 n-6	0.31 ± 0.00	0.33 ± 0.01	0.47 ± 0.01 [a #]	0.32 ± 0.01 [b]
20:4 n-6	11.22 ± 0.06	11.09 ± 0.05	9.70 ± 0.25 [#]	9.70 ± 0.26 [#]
22:4 n-6	3.41 ± 0.02	3.30 ± 0.02	3.08 ± 0.09 [#]	3.23 ± 0.06
22:5 n-6	1.22 ± 0.05 [b]	1.64 ± 0.10 [a]	0.91 ± 0.07 [b #]	1.09 ± 0.02 [a #]
18:3 n-3	0.03 ± 0.01	0.04 ± 0.01	0.27 ± 0.00 [a #]	0.04 ± 0.03 [b]
22:5 n-3	0.17 ± 0.01	0.13 ± 0.01	0.51 ± 0.14 [a #]	0.23 ± 0.01 [b]
22:6 n-3	10.51 ± 0.16 [a]	9.54 ± 0.17 [b]	8.64 ± 0.05 [#]	8.72 ± 0.04 [#]
∑ PUFA	28.20 ± 0.32 [a]	27.02 ± 0.25 [b]	26.13 ± 0.51 [#]	25.43 ± 0.72 [#]
∑ n-3	10.71 ± 0.15 [a]	9.71 ± 0.17 [b]	9.45 ± 0.05 [a #]	8.99 ± 0.17 [b #]
∑ n-6	17.43 ± 0.09	17.67 ± 0.02	16.70 ± 0.50	16.44 ± 0.82
∑ trans	n.d. [b]	0.18 ± 0.00 [a]	n.d. [b]	0.43 ± 0.08 [a #]
n6/n3 ratio	1.62 ± 0.00	1.81 ± 0.00	1.77 ± 0.03	1.82 ± 0.03

The following fatty acids were found at concentrations lower than 0.5% and for this reason are not shown: C14:0, C15:0, C16:1 n7, C17:0, C20:0, C18:3 n-3, C20:3 n6, C22:0, C22:1 n-6, C22:1 n-9, C24:0 e C24:1 n-9. The following fatty acids were not detected in the analyzed samples: C20: n-3 e C22:2 n-6.

Lowercase indicate significant difference among SO/FO and HVF groups ($P<0.05$).

indicate significant difference among different period (gestation or lactation) in the same supplemented group ($P<0.05$).

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.


SO/FO
HVF
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CMS
Gestation
Lactation
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Recognition Index - 24 h
1.00
0.75
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Lactation

Figure 1

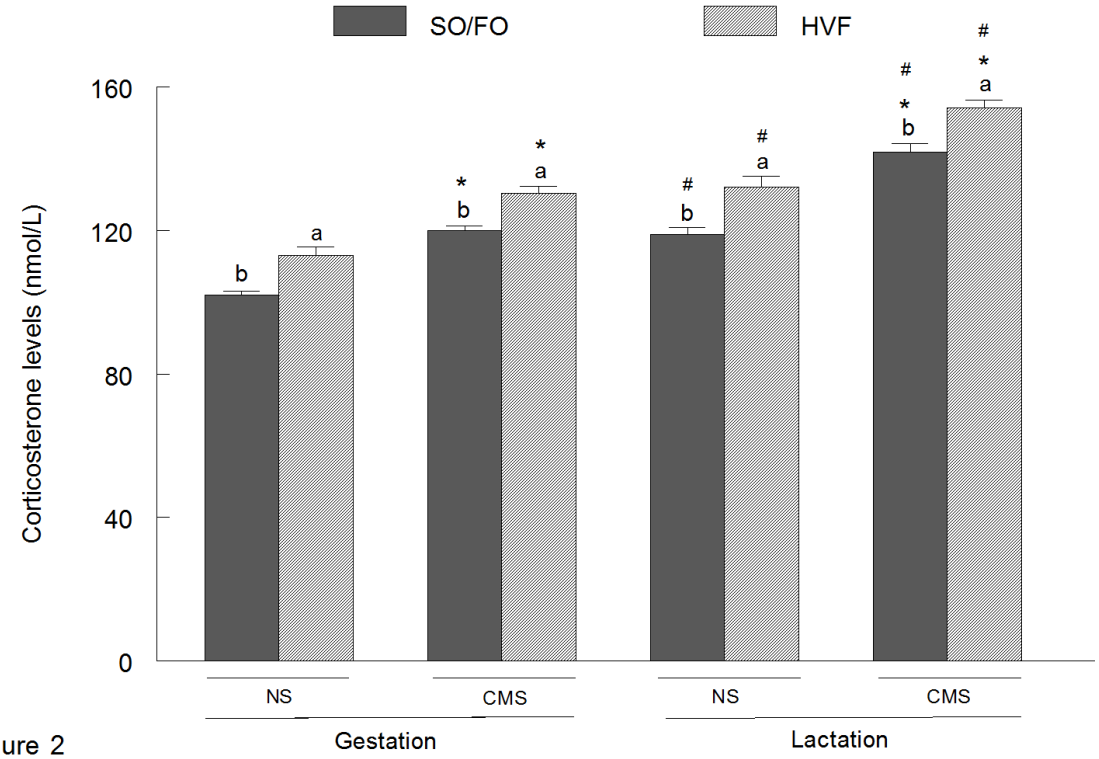
SO/FO
HVF
Corticosterone levels (nmol/L)
160
120
80
40
0
b
a
*
b
*
a
#
b
#
a
#
*
b
#
*
a
NS
CMS
NS
CMS
Gestation
Lactation

Figure 2

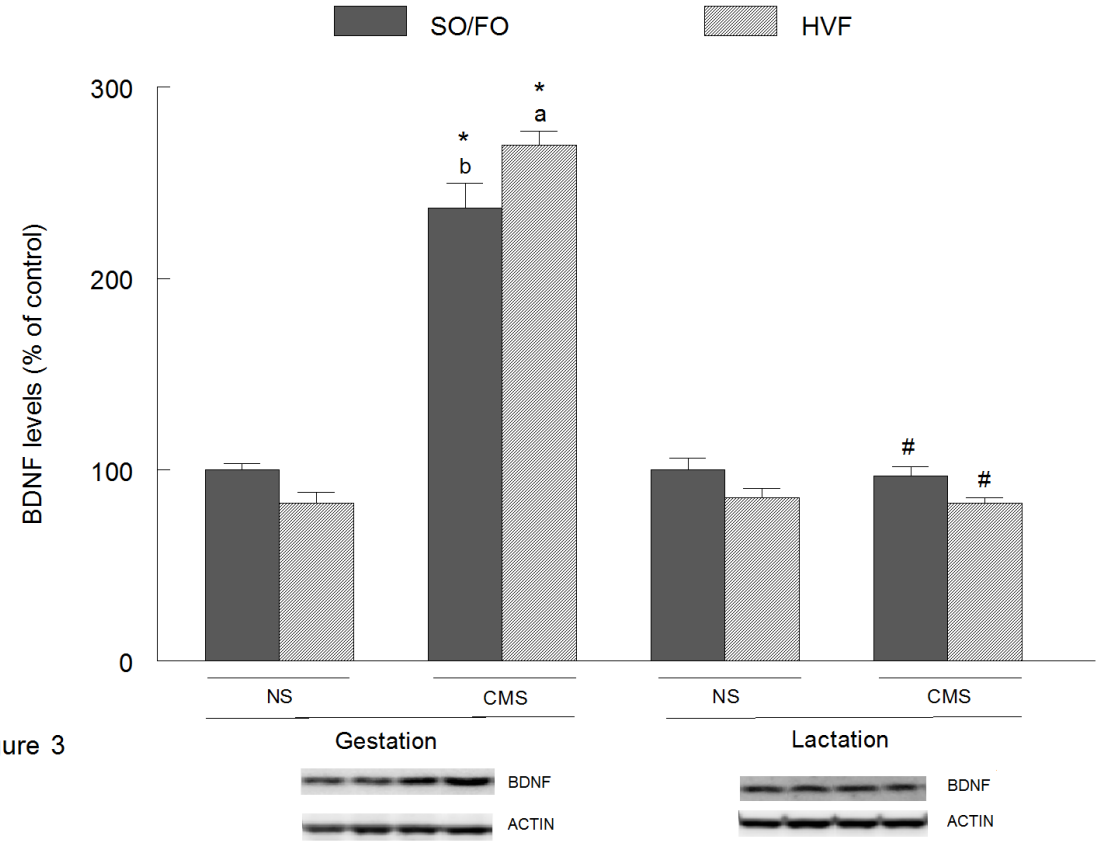
SO/FO
HVF
BDNF levels (% of control)
300
200
100
0
*
b
*
a
#
#
NS
CMS
NS
CMS
Gestation
Lactation
BDNF
ACTIN
BDNF
ACTIN

Figure 3

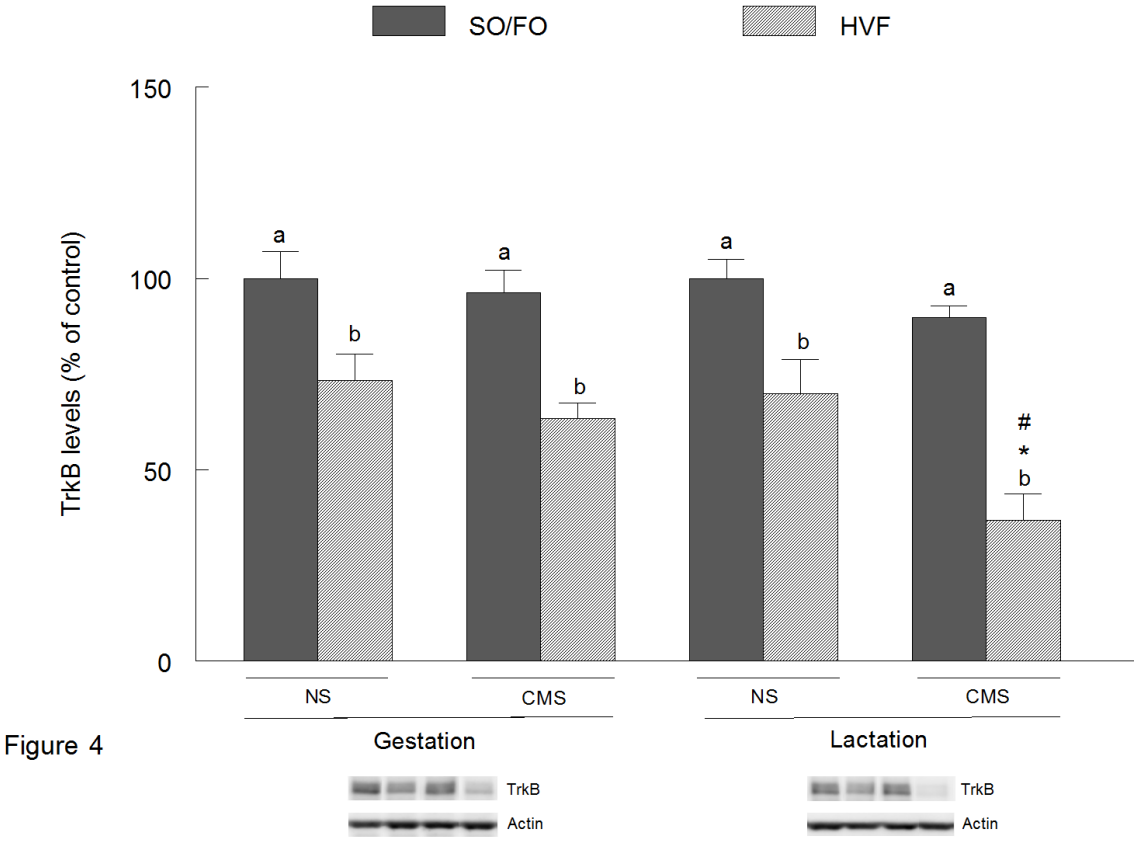
SO/FO
HVF
150
100
50
0
TrkB levels (% of control)
a
b
a
b
a
b
a
#
*
b
NS
CMS
NS
CMS
Gestation
Lactation
TrkB
Actin
TrkB
Actin

Figure 4

5 DISCUSSÃO

A nutrição, principalmente durante o período perinatal, exerce influência decisiva no desenvolvimento e na manutenção das funções psicomotoras e cognitivas no decorrer da vida e embora todos os nutrientes sejam importantes para o desenvolvimento estrutural do sistema nervoso central (SNC), alguns lipídeos, como os ácidos graxos, podem influenciar decisivamente o desenvolvimento neuronal (GEORGIEFF, 2006). Como a mãe é a principal fonte nutricional do feto e recém-nascido (BUCKLEY et al., 2005), deve-se considerar não só a quantidade, mas o tipo de ácido graxo materno consumido durante a gestação e lactação (STORLIEN et al., 1991; INNIS, 2007), uma vez que é durante o desenvolvimento embrionário e pós-natal que ocorrem os processos de neurodesenvolvimento mais importantes - mielinização, organização de sistemas neurotransmissores, arborização dendrítica e gênese sináptica (SALIBA e MARRET, 2001). Neste momento, o cérebro é especialmente vulnerável a quaisquer deficiências ou alterações nutricionais, em função de ser o período em que os processos implicados no desenvolvimento cerebral ocorrem com maior rapidez (ALMEIDA et al., 2002; MORGANE et al., 2002).

Sendo assim, a partir dos resultados apresentados no artigo 1, foi possível observar os efeitos benéficos do consumo de uma dieta enriquecida com azeite de oliva em diferentes fases do desenvolvimento, como gestação, lactação ou após o desmame dos filhotes até a idade adulta. O azeite de oliva é um componente essencial na dieta Mediterrânea tradicional, a qual já foi associada a reduzido risco de problemas cognitivos relacionados ao envelhecimento (SOLFRIZZI et al., 1999) e proteção contra a doença de Alzheimer (PANZA et al., 2004), bem como controle do sobrepeso e obesidade (SCHRODER et al., 2004, SORIGUER et al., 2009).

No presente estudo, contatou-se alteração no peso corporal e estresse oxidativo em todos os períodos analisados após o consumo da dieta contendo azeite de oliva. Foi observada também a influência da dieta, somente durante o período perinatal, sobre a expressão de fatores neurotróficos, com significativo aumento do BDNF, bem como os seus principais exons IV e VI e FGF-2. Esta influência benéfica tem sido atribuída ao favorável perfil de ácidos graxos presentes no azeite de oliva, além dos compostos polifenóis com potencial antioxidante que em conjunto podem contribuir para a manutenção da integridade estrutural das membranas neuronais (PANZA et al., 2004). Isso sugere que o consumo de uma dieta enriquecida com azeite de oliva ao longo da vida pode produzir alterações persistentes sobre o estado oxidativo, bem como na expressão de fatores neurotróficos, exercendo efeitos benéficos especialmente importantes no desenvolvimento do sistema nervoso central.

Seguindo a linha de raciocínio de que os ácidos graxos presentes na dieta, principalmente durante períodos iniciais do desenvolvimento podem causar alterações que são observadas ao longo da vida, nós decidimos avaliar a influência da alteração nos hábitos alimentares observados nas últimas décadas, principalmente na sociedade Ocidental, com o elevado consumo de alimentos processados, ricos em ácidos graxos *trans*. Neste contexto, o segundo e terceiro estudos avaliam os efeitos do consumo prolongado de gordura vegetal hidrogenada (GVH), rica em ácidos graxos *trans* (AGT), sobre o sistema nervoso central.

A partir destes experimentos, foi possível observar que o consumo ou suplementação de GVH, rica em ácidos graxos *trans*, em diferentes fases do desenvolvimento foi capaz de precipitar a ocorrência de comportamento hiperativo, o qual é um dos comportamentos que caracterizam o Transtorno do Déficit de Atenção e Hiperatividade (TDAH). Nossos resultados mostraram que a ingestão prolongada de gordura *trans* por 10 meses, bem como durante a gestação e lactação ao longo de uma ou duas gerações de animais, foi capaz de exacerbar o comportamento hiperativo destes animais. A fisiopatologia precisa dos sintomas de hiperatividade presentes no TDAH ainda é desconhecida, porém tem-se demonstrado o envolvimento da dopamina (LAHOSTE et al., 1996). Como o cérebro necessita de aporte adequado de ácidos graxos para a manutenção da sua integridade estrutural, e consequentemente suas funções normais, nós acreditamos que a presença dos ácidos graxos *trans* nas membranas neuronais pode prejudicar a fluidez e plasticidade sináptica, afetando a neurotransmissão dopaminérgica, as quais podem predispor o aumento do comportamento de hiperatividade. Outro fator importante a ser considerado, é que o alto consumo de alimentos industrializados ricos em AGT, principalmente entre as crianças, leva a uma redução na ingestão de AGPI, como o DHA. Em humanos, já foi relatado que a deficiência de AGPI, bem como menor conteúdo de DHA no cérebro, também predispõe crianças ao desenvolvimento de TDAH (BENTON, 2010; SINN, 2008; SINN, BRYAN, e WILSON, 2008), porém até o momento, o nosso estudo é o primeiro a mostrar uma ligação clara entre o consumo crônico de gordura *trans* e o desenvolvimento de sintomas de hiperatividade.

Dando continuidade ao nosso trabalho, nós decidimos investigar se a incorporação de ácidos graxos *trans* nas membranas neuronais depende do período de desenvolvimento em que ocorre a suplementação com gordura *trans*, e se tais alterações podem causar respostas diferentes na idade adulta após exposição a estresse. Adicionalmente às observações comportamentais de memória, este estudo inclui algumas análises moleculares, as quais foram desenvolvidas em busca de mecanismo mais específico que possa explicar os eventos aqui mostrados. Sendo assim, tanto a suplementação com uma razão ideal de ácidos graxos (óleo de

soja/óleo de peixe) ou GVH mostrou que os ácidos graxos são incorporados de maneira diferente dependendo do período de suplementação, onde se observou maiores níveis dos metabólitos dos AG das séries n-3 e n-6 (DHA, 22:6 n-3 e ARA, 20:4 n-6) na gestação, enquanto que na lactação, houve maior incorporação dos precursores ALA (18:3 n-3) e LA (18:2 n-6), respectivamente. Em contrapartida, os AGT foram mais incorporados durante o período da lactação somente no grupo GVH, confirmando que a barreira placentária pode apresentar seletividade na passagem dos AG pra o feto (DUTTAROY, 2009; HANEBUTT et al., 2008; HERRERA et al., 2006). Como os ácidos graxos são nutrientes fundamentais para o desenvolvimento fetal e pós-natal (HERRERA et al., 2006; INNIS 2006), estes resultados sugerem que não somente o tipo de ácido graxo, mas também o período de desenvolvimento, em que ocorre a suplementação é muito importante, uma vez que ambos podem causar alterações específicas no perfil lipídico das membranas neuronais, produzindo consequências adversas ao longo da vida (HERRERA, 2002). Neste mesmo estudo, mostramos que a presença de AGT no hipocampo foi relacionada ao prejuízo da memória de curta e longa duração juntamente com menores níveis de receptores TrkB, resultados estes obtidos em animais expostos ou não ao estresse. Além disso, os níveis de BDNF também foram alterados pela suplementação com GVH e exposição ao estresse.

Como o hipocampo é a principal região do cérebro associada com a memória (MARTIN e CLARK, 2007), acreditamos que a variação na composição dos AG observada neste estudo pode estar correlacionada com danos cognitivos na idade adulta de prole. Alguns trabalhos mostram que alterações na composição dos ácidos graxos, bem como a incorporação de AGT nas membranas neuronais podem afetar a plasticidade sináptica (LARQUÉ et al., 2003), a qual modifica a neurotransmissão e causa alterações em proteínas ligadas à membrana. Neste sentido, nós acreditamos que a incorporação significativa de AG *trans* nas membranas do hipocampo levou a uma redução dos níveis dos receptores TrkB que resultou em maior prejuízo da memória nos animais suplementados com GVH. Estes resultados estão de acordo com outros estudos, onde a redução dos níveis do receptor TrkB contribui para a perda de memória (KEMPPAINEN et al., 2012). Da mesma maneira, a variação nos níveis de BDNF após a exposição ao estresse crônico encontrada nos nossos resultados mostra que o aumento dos níveis de BDNF foi responsável por melhorar a memória de curto prazo nos filhotes adultos suplementados com OS/OP durante o período da gestação, entretanto a sua superexpressão observada no grupo suplementado com GVH resultou em déficits da função cognitiva. Estes dados estão de acordo com outros autores, onde o aumento do BDNF foi relacionado com a melhora da memória (ALOMARI et al., 2013; LIU et al., 2014), enquanto que déficits de

aprendizagem e deficiência na memória foram observadas após a superexpressão de BDNF no sistema nervoso central.

Evidências sugerem que o desenvolvimento e a manutenção das funções do sistema nervoso central, no decorrer da vida, sofrem influência decisiva da nutrição principalmente no período perinatal. Tomados em conjunto com os dados apresentados nesta tese, é possível sugerir que a ingestão de alimentos saudáveis durante o início do desenvolvimento, como a dieta Mediterrânea a qual inclui o consumo de azeite de oliva, é capaz de agir positivamente sobre o estado oxidativo cerebral e fatores neurotróficos, contribuindo assim favoravelmente para diminuir a incidência e gravidade de condições neuropsiquiátricas. Por outro lado, o aumento do consumo de alimentos processados, ricos em AG *trans*, em detrimento de ácidos graxos essenciais para o desenvolvimento do SNC, principalmente da família n-3, pode aumentar a suscetibilidade ao desenvolvimento de desordens neuropsiquiátricas, entre elas a hiperatividade associada ao TDAH e prejuízos de memória.

6 CONCLUSÃO

Através dos resultados obtidos podemos chegar às seguintes conclusões:

- ✓ O consumo de uma dieta enriquecida com azeite de oliva, o qual apresenta perfil lipídio e antioxidante favorável, durante períodos iniciais do desenvolvimento melhorou o status oxidativo cerebral de ratos, bem como aumentou a expressão de fatores neurotróficos importantes para a manutenção das funções do sistema nervoso central.
- ✓ O consumo prolongado de gordura vegetal hidrogenada (rica em ácidos graxos *trans)* após o desmame até a idade adulta e ao longo de duas gerações de ratos, foi associado a um comportamento hiperativo dos animais, o que pode indicar uma predisposição ao desenvolvimento do transtorno de déficit de atenção e hiperatividade, que por analogia, tem aumentado nas últimas décadas.
- ✓ A suplementação com gordura vegetal hidrogenada durante a lactação permitiu maior incorporação de AG *trans* em comparação ao período da gestação, mostrando que o período do desenvolvimento em que ocorre a oferta dos diferentes lipídeos através da placenta ou leite materno pode ser determinante na formação do perfil lipídico das membranas neurais. A incorporação de AG *trans* no hipocampo modificou os níveis de marcadores moleculares que refletiram em prejuízos na função cognitiva em ratos expostos ou não ao estresse, sendo estes danos mais intensos nos animais suplementados durante a lactação em comparação com o período da gestação.

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