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INFLUÊNCIA DOS ÁCIDOS GRAXOS DA DIETA SOBRE PARÂMETROS DE ADICÇÃO À ANFETAMINA EM RATOS: ASPECTOS COMPORTAMENTAIS, BIOQUÍMICOS E MOLECULARES

TESE DE DOUTORADO

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por

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

Orientadora: Profª. Drª. Marilise Escobar Bürger

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria

INFLUÊNCIA DOS ÁCIDOS GRAXOS DA DIETA SOBRE PARÂMETROS DE ADICÇÃO À ANFETAMINA EM RATOS: ASPECTOS COMPORTAMENTAIS, BIOQUÍMICOS E MOLECULARES

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Data e Local da Defesa: Santa Maria, 16 de julho de 2015.

Ácidos graxos poliinsaturados (AGPI) são constituintes das membranas fosfolipídicas neuronais onde exercem funções fundamentais para o desenvolvimento e funcionamento do cérebro. As últimas décadas foram acompanhadas de mudanças nos hábitos alimentares, especialmente em países ocidentais, devido à industrialização dos alimentos, o que contribuiu para o consumo aumentado de ácidos graxos *trans* (AGT) e ácidos graxos ômega-6 (AG n-6) em detrimento de AG ômega-3 (n-3). Recentes estudos do nosso grupo tem mostrado que tais mudanças alimentares podem modificar a composição das membranas fosfolipídicas neurais, modificando o sistema dopaminérgico, o que pode facilitar a preferência por drogas aditivas psicoestimulantes. O presente estudo foi conduzido através de dois protocolos experimentais: 1) Ratas Wistar adultas, divididas em quatro grupos experimentais, foram suplementadas diariamente com óleo de soja (OS, rico em AGPI n-6), óleo de peixe (OP, rico em AGPI n-3) ou gordura vegetal hidrogenada (GVH, rica em AGT) e grupo controle (suplementados com água), desde o período pré-concepcional até o desmame da 2ª geração, cujos filhotes permaneceram nas mesmas suplementações até os 40 dias pós-natal. Ratos machos nascidos a partir da 2ª geração foram condicionados ao protocolo de preferência com anfetamina (ANF), e posteriormente submetidos à protocolos de observação comportamental de preferência à droga, desenvolvimento de ansiedade, avaliação do perfil lipídico dos tecidos cerebrais, bem como parâmetros de estresse oxidativo nas mesmas áreas cerebrais; 2) Ratas Wistar adultas, designadas em três grupos experimentais, foram diariamente suplementadas com uma mistura de OS e OP gerando uma razão ideal de AGPI n-6:n-3 (2:1) ou GVH, e grupo controle (suplementados com água), desde o período pré-concepcional até o desmame da 1ª geração, cujos filhotes machos foram mantidos sob as mesmas suplementações das progenitoras até os 50 dias pós-natal, quando foram submetidos à um protocolo de autoadministração de ANF,

seguido de avaliações comportamentais, análises moleculares e perfil de incorporação de AG em diferentes áreas cerebrais. Animais de 2ª geração suplementados com GVH apresentaram incorporação de AGT em regiões cerebrais relacionadas à adicção, e maior preferência por ANF, indicando o envolvimento de alterações no circuito dopaminérgico. Animais de 1ª geração suplementados com GVH apresentaram maior frequência de autoadministração de ANF, indicando maior grau de adicção pelo psicoestimulante. Além dos animais suplementados com GVH de ambos protocolos experimentais terem apresentado maior grau de ansiedade, as duas gerações de ratos apresentaram maior estatus oxidativo, acompanhados de modificações moleculares em áreas dopaminérgicas cerebrais. Os resultados mostram que o consumo crônico de gorduras *trans*, em detrimento de ácidos graxos poliinsaturados principalmente da série n-3, pode modificar a constituição lipídica das membranas neuronais e afetar sua plasticidade, modificando a neurotransmissão dopaminérgica, a qual está fortemente associada ao desenvolvimento de adicção por drogas psicoestimulantes, além de ansiedade que é um dos sintomas da dependência pela ANF.

Palavras-chave: Ácidos graxos *trans*, anfetaminas, ômega-3, drogas de abuso, adicção

ABSTRACT

Doctoral Thesis
Graduate Program in Pharmacology
Federal University of Santa Maria

INFLUENCE OF DIFFERENT DIETARY FATTY ACIDS ON AMPHETAMINE ADDICTION PARAMETERS IN RATS: BEHAVIOR, BIOCHEMICAL AND MOLECULAR

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Date and place of defense: July 16th, 2015, Santa Maria

Polyunsaturated fatty acids (PUFA) are constituents of neuronal membrane phospholipids which play key roles in the development and functioning of the brain. The last decades have been accompanied by changes in dietary habits, especially in Western countries through the industrialization of foods, which contributed to the increased consumption of *trans* fatty acids (TFA) and omega-6 fatty acid (FA n-6) over AG omega-3 (n-3). Recent studies of our group have shown that such dietary changes can alter the composition of neuronal membrane phospholipids, altering the dopaminergic system, which may facilitate the preference for psychostimulant addictive drugs. This study was conducted through two experimental protocols: 1) adult Wistar rats were divided into four experimental groups were daily supplemented with soybean oil (SO rich in PUFA n-6), fish oil (FO rich in PUFAs n-3) or hydrogenated vegetable fat (HVF, rich in TFAs) and control group (supplemented with water), from pre-conception period until weaning of the 2nd generation, whose offspring remained the same supplements until 40 post-natal day (PND). Male rats born from the 2nd generation were conditioned in protocol preference with amphetamine (AMPH), and subsequently under behavioral observation rather than for drug development of anxiety, assessment of lipid profile of brain tissue, as well as parameters of stress oxidative the same brain areas; 2) adult Wistar rats, divided into three experimental groups were supplemented daily with a mixture of SO and FO which was generating an ideal ratio of PUFA n-6/n-3 (2:1) or HVF and control group (supplemented with water), since the pre-conceptional period until weaning of 1st generation, whose male pups were kept under the same progenitor supplements until 50 post-natal day when they were submitted to a protocol of self-administration of AMPH, followed by behavioral assessments, analysis and molecular incorporation of FA profile in different brain areas. Animals supplemented by 2nd generation HVF showed incorporation of TFA in whole brain, and increased preference for AMPH, indicating the involvement of

changes in dopaminergic circuitry. Animals supplemented with HVF 1st generation had a higher frequency of self-administration of AMPH, indicating greater degree of addiction by psychostimulant. The animals supplemented with HVF in both experimental protocols showed a higher degree of anxiety, the two generations of rats showed increased oxidative status, accompanied by molecular damage in dopaminergic brain areas. The results show that chronic consumption of *trans* fats at the expense of polyunsaturated fatty acids especially the n-3 series, may modify the constitution of lipid membranes and affect neuronal plasticity, modifying dopaminergic neurotransmission, which is strongly associated with the development of addiction by psychostimulant drugs, and anxiety which is one of the symptoms of AMPH-addiction.

Key-words: *Trans* fatty acids, amphetamine, omega-3, addiction

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LISTA DE ABREVIATURAS E SIGLAS

AA - ácido araquidônico / araquidonic acid

ADHD – attention déficit hyperactivity disorder

AG - ácido graxo(s)

AGE - ácidos graxos essenciais

AGPI - ácido(s) graxo(s) poliinsaturado(s)

AGT - ácido(s) graxo(s) trans

ALA - ácido α-linolênico

AMPH - amphetamine(s)

ANF - anfetamina

BDNF - brain derived neurothrophic factor

CNS - central nervous system

CPP - conditioned place preference

D1R - dopamine D1 receptor

D2R - dopamine D2 receptor

DA - dopamine / dopamina

DAT - dopamine transporter

DHA - ácido docosahexaenóico /docosahexaenoic acid

EPM - elevated plus maze

FA - fatty acid(s)

FO - fish oil

FR - fixed ratio

HVF - hydrogenated vegetable fat

LA - ácido linoleico

MET - metanfetamina

OF - open field

OP - óleo de peixe

OS - óleo de soja

PCL - preferência condicionada de lugar

PND - post-natal day

PUFA - polyunsaturated fatty acid(s)

RDC - resolução da diretoria colegiada

SNC - sistema nervoso central

SO - soybean oil

TDHA – transtorno do déficit de atenção e hiperatividade

TFA - trans fat acid(s)

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APRESENTAÇÃO

Esta tese está estruturada em seções disposta da seguinte forma: Introdução, Objetivos, Artigos Científicos, Discussão, Conclusões, Perspectivas e Referências Bibliográficas.

Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se inseridos no próprio artigo na seção **ARTIGOS CIENTÍFICOS**, composta por dois artigos publicados, completando assim a íntegra deste estudo.

Ao fim desta tese encontra-se o item **CONCLUSÕES FINAIS**, nos quais há interpretações e comentários gerais sobre o estudo.

As referências referem-se às citações que aparecem no item **INTRODUÇÃO DISCUSSÃO** e **CONCLUSÕES** desta tese.

1 INTRODUÇÃO

1.1 O consumo de diferentes ácidos graxos e a influência sobre o desenvolvimento cerebral

A composição nutricional durante o período de formação do cérebro é o principal determinante da capacidade funcional cerebral e da plasticidade das membranas neuronais durante a idade adulta (TYAGI et al., 2015; VAN DE REST et al., 2012; MEGUID et al., 2008; GOMEZ-PINILLA, 2008; INNIS, 1991). A capacidade funcional do cérebro corresponde a habilidade de executar as tarefas como as de cognição e movimento, e a plasticidade refere-se a rigidez e permeabilidade das membranas que é determinada principalmente pela composição dos ácidos graxos presentes (BOURRE, 2004; YEHUDA, RABINOVITZ & MOSTOFSKY, 1999). Durante a fase pré-natal e amamentação, os ácidos graxos (AG) da gestante são transferidos ao feto, de forma que há uma grande incorporação desses AG e consequente maturação das células neuronais nesse período (JONES et al., 2013; INNIS, 2007). Os principais AG constituintes das membranas neuronais são os ácidos graxos poliinsaturados (AGPI) sendo principalmente das séries ômega-3 (n-3), na forma de ácido docosahexaenóico (DHA), e os da série ômega-6 (n-6), principalmente na forma de ácido araquidônico (AA) (BOURRE, 2004; YEHUDA et al., 2005). Os AGPI, para os mamíferos, são obtidos exclusivamente da dieta, os n-3 consumidos na forma de ácido α-linolênico (ALA) proveniente principalmente de peixes e frutos do mar, e os n-6 consumidos na forma de ácido linoleico (LA) que provém na maior parte de óleos de sementes como a soja, o girassol e o milho (HULBERT et al., 2005; WAINWRIGHT, 1992; DZIEZAK, 1989).

Nas últimas décadas ocorreu uma mudança nos hábitos alimentares, levando a um aumento do consumo de AG n-6 em detrimento de n-3, por ser o n-6 um AG presente nos óleos mais utilizados atualmente na culinária em todo mundo, modificando assim o perfil de ingesta da proporção de AG n6:n3, que no passado era de 1:1 a 3:1, atualmente chega a 25:1 (SIMOPOULOS, 2011; CORDAIN et al., 2005; SIMOPOULOS, 1999). O impacto destas alterações na relação de AGPI no neurodesenvolvimento depende do estágio de desenvolvimento e duração do tempo de consumo, evidências mostram que há um efeito de

acumulação durante gerações, indicando que a incorporação é tempo dependente (TREVIZOL et al., 2013; KUHN et al., 2013). O último trimestre de gestação é uma fase determinante para completar o desenvolvimento fetal do cérebro e a neurogênese, pois durante essa fase são necessárias grandes quantidades de n-3 e n-6 para a síntese de fosfolípidios da membrana, e apesar dos estudos realizados, ainda são desconhecidos os efeitos da mudança no perfil de AG sobre o desenvolvimento do cérebro (KAWAKITA et al., 2006; INNIS E DE LA PRESA OWENS, 2001).

O alto consumo de alimentos industrializados que contém grandes quantidades de ácidos graxos *trans* (AGT) também é responsável pela modificação do perfil lipídico cerebral, além dos mesmos terem a capacidade de incorporação. Os AGT presentes nos alimentos derivam de duas diferentes fontes: industrial e natural (SANTOS, CRUZ & CASAL, 2015; ARCAND et al., 2014), AGT de fonte natural são produzidos como um resultado da transformação microbiana de AG insaturados em animais ruminantes e assim existem em níveis baixos em alguns produtos, tais como produtos lácteos, não representando risco à saúde BHARDWAJ, PASSI & MISRA, 2011). O grande problema dos AGT reside em alimentos processados onde são adicionados AGT industriais, esses AGT são produzidos por hidrogenação parcial de óleos vegetais. A adição desses AGT nos alimentos processados tem por objetivo aumentar a sua estabilidade elevando assim o tempo de prateleira desses alimentos, por ser uma molécula altamente estável e ter um alto ponto de fusão e pela característica de se formar uma molécula que possui a insaturação com carbonos ligados a hidrogênios em posição *trans*, ao contrário dos ácidos graxos *cis* que possuem os hidrogênios no mesmo lado da cadeia na insaturação (Figura 1). Além disso, esses AGT são capazes de proporcionar uma maior crocância e palatabilidade aos alimentos, tornando-se um fator que confere maior valor comercial e eleva o consumo desses alimentos, dificultando a retirada ou substituição desse componente pelas indústrias. Já estão bem estabelecidos e conhecidos os efeitos danosos dos AGT industriais a nível periférico no organismo, causando alterações prejudiciais nos níveis de lipídios plasmáticos (elevando o colesterol através do aumento do nível de lipoproteínas de baixa densidade – LDL, do inglês *low density lipoprotein* e diminuindo as lipoproteínas de alta densidade – HDL, do inglês *high density lipoprotein*), promovendo uma inflamação sistêmica, resultando em adiposidade visceral e resistência à insulina, contribuindo de forma significativa para um aumento de dano cardiovascular (MOZAFFARIAN & WILLETT, 2007), porém os efeitos a nível de cérebro e membranas neuronais ainda são pouco estudados.

Desde 2003, a Resolução brasileira nº 360 (BRASIL, RDC nº 360/ 2003) exigiu que os fabricantes de alimentos processados divulgassem o conteúdo de gorduras *trans* por porção no

rótulo com a descrição nutricional dos componentes. Porções recomendadas foram estabelecidas pela Resolução 359 (BRASIL, RDC nº 359/2003). A referida resolução definiu o tamanho da porção baseada na quantidade média de alimentos que devem ser consumidos por pessoas saudáveis com idade de 36 meses ou mais para promover uma dieta saudável. Porém o consumo por porção que foi determinado pela legislação muitas vezes não condiz com o consumo médio da população, sendo que a quantidade consumida de AGT pode ser muito maior do que se estima. De acordo com a Resolução 360, as empresas podem fazer alegações de "zero gorduras *trans*" ou "livre de gorduras *trans*" nas embalagens no caso da quantidade de gorduras *trans* por porção contiver até 0,2 gramas, uma quantidade considerada insignificante pela resolução. Assim, se a quantidade de AGT é de até 0,2 gramas por porção, uma empresa não precisa informar que o alimento contém gorduras *trans* nas informações nutricionais do rótulo, por isso é impossível conhecer o conteúdo de AGT na maioria dos produtos. 40% a 60% dos alimentos industrializados vendidos em supermercados brasileiros que relatam "zero de gorduras *trans*" ou "livre de gorduras *trans*" em suas embalagens contém gorduras *trans* (SILVEIRA, GONZALEZ-CHICA & PROENÇA, 2013).

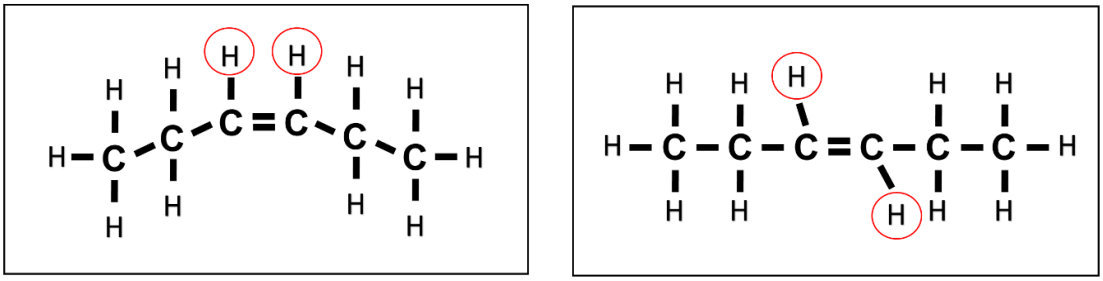

Figura 1. Ácido graxo *cis* (à esquerda) e *trans* (à direita).

1.2 O sistema dopaminérgico, substâncias que alteram seu funcionamento e marcadores da sua atividade

A dopamina (DA) é o neurotransmissor catecolaminérgico predominante no cérebro dos mamíferos, onde controla diversas funções, incluindo a atividade locomotora, cognição, emoção, sistemas de recompensa e o apetite (MISSALE et al., 1998). Foram identificados cinco tipos de receptores dopaminérgicos, dois deles (D1 e D5) acoplados a proteína Gs que atuam ativando a adenilato ciclase, e os outros três (D2, D3 e D4) acoplados a proteína Gi, que inibem a adenilato ciclase.

O sistema dopaminérgico mesolímbico está envolvido nos mecanismos de recompensa e reforço de drogas, como ocorre com o uso de psicoestimulantes e outras drogas que aumentam a liberação de DA nesse sistema, que envolve principalmente as seguintes áreas cerebrais: hipocampo, área tegmentar ventral, amígdala, núcleo accumbens e córtex. Sabe-se que ambos os receptores D1 e D2 estão ativados durante o reforço na autoadministração de drogas psicoestimulantes (SHI & MCGINTY, 2011), a ativação dos receptores D1 tem um papel sobre a atividade de busca pela droga, enquanto que os receptores D2 regulam os efeitos de recompensa provocados pelo reforço (PHILLIPS, ROBBINS & EVERITT, 1994; MALDONADO et al. 1993; BENINGER, HOFFMAN & MAZURSKI, 1989).

O fator neurotrófico derivado do encéfalo (BDNF – do inglês *brain derived neurotrophic factor*) é o fator neurotrófico mais abundante produzido no cérebro dos mamíferos, o qual promove o crescimento e o desenvolvimento dos neurônios. Tem um papel essencial no estabelecimento do número adequado de neurônios dopaminérgicos no sistema nervoso central (SNC) (BAQUET et al.; 2005). Estudos mostram uma modulação do BDNF corticoestriatal através da suplementação com APGI n-3, sugerindo uma associação entre a concentração desses AGs e do BDNF (BOUSQUET et al.; 2009). Numerosos estudos tem mostrado que o BDNF também age no efeito de recompensa associado ao uso de psicoestimulantes tendo um papel modulador no sistema dopaminérgico, atuando como um fator importante que influencia a plasticidade estrutural das membranas associado com a dependência pelas drogas (LANG, 2007; TYAGI et al., 2014).

Fazendo parte do sistema dopaminérgico, o transportador de dopamina (DAT - do inglês *dopamine transporter*) localiza-se na porção pré-sináptica dos terminais nervosos dopaminérgicos e possui a função de regular a neurotransmissão da DA, controlando as

concentrações de DA disponíveis para a ligação nos receptores pré e pós-sinápticos através da sua recaptação aos terminais pré-sinápticos (AMARA & KUHAR, 1993). O DAT está localizado na bicamada fosfolipídica, e as alterações da constituição das membranas celulares neuronais podem alterar a sua função, como ocorre quando há uma deficiência de AGPI (BOURRE et al., 1989; KODAS et al., 2002).

AGPI estão funcionalmente envolvidos com vários sistemas de neurotransmissão, incluindo o monoaminérgico (CHALON, 2006; ZIMMER et al., 2000). Níveis diminuídos de AGPI n-3 durante o desenvolvimento do cérebro podem prejudicar a função dos sistemas serotoninérgico e dopaminérgico em regiões corticais e subcorticais, modificando vários aspectos do comportamento (YAVIN., 2006; LEVANT et al., 2004), e contribuindo para a etiologia de algumas doenças neurológicas, como o desenvolvimento de mania e distúrbio bipolar (MCNAMARA & CARLSON, 2006). O reduzido consumo de DHA pode produzir um efeito significativo através da redução da densidade de receptores D2 no estriado ventral. Ratas com diminuição de n-3 também apresentaram maior densidade de receptores D1 no núcleo caudado do que as ratas com DHA normal (DAVIS et al. 2013). Estas alterações nos receptores são semelhantes as que são encontradas em vários modelos de depressão em roedores (PASSOS et al., 2012).

Acar et al. (2003) mostrou que o consumo de AGT ou uma dieta desequilibrada em AG n-6/n-3 pode atuar sobre os níveis de neurotransmissores dopaminérgicos no estriado e no hipocampo, através da avaliação dos metabólitos da DA. Porém não existem estudos moleculares envolvendo o sistema dopaminérgico e o consumo de AGT ou de dietas com diferentes razões de AGPI.

Como o sistema nervoso central têm necessidades nutricionais básicas de AGPI, muitos fatores são associados com a deficiência nesses AG e o aumento da vulnerabilidade para transtornos afetivos e impulsivos. Sabe-se que a diminuição na ingesta de AGPI pode aumentar a vulnerabilidade a distúrbios de dependência por drogas psicoestimulantes e agressão em humanos (BUYDENS-BRANCHEY, BRANCHEY & HIBBELN, 2008). O abuso de drogas psicoestimulantes é um grave problema de saúde pública em todo o mundo pelo alto poder de adicção e desenvolvimento de complicações neuropsiquiátricas.

As drogas psicoestimulantes que possuem maior efeito de adicção são as anfetaminas (ANF). O número de novos usuários de ANF, principalmente metanfetamina, entre as pessoas a partir dos 12 anos foi de 144.000 em 2013 (SAMHSA, 2014). O uso de medicamentos psicoestimulantes derivados das ANF prescritos para fins não-médicos continua sendo uma parte significativa do problema do abuso de drogas na adolescência. No mesmo ano, 15% dos

adolescentes do ensino médio haviam utilizado algum medicamento de prescrição para fins não terapêuticos. Um levantamento realizado pelo National Institute on Drug Abuse (NIDA) mostra que há o abuso de psicoestimulantes como o Adderall® (DL-anfetamina), comumente usado para tratar transtorno de déficit de atenção e hiperatividade (TDAH), com 7,4% de incidência, e de abuso de Ritalina® (metilfenidato) sendo de 2,3%, outro medicamento utilizado no TDAH (NIDA, 2014).

O mecanismo de ação das ANF está principalmente relacionado ao sistema dopaminérgico, promovendo o aumento da liberação neuronal de DA nos neurônios pré-sinápticos, além da inibição da sua recaptação pelo DAT, que apresenta papel fundamental no equilíbrio da atividade do sistema dopaminérgico, noradrenérgico e serotoninérgico, e bloqueio da ação da enzima responsável pela regulação das monoaminas na fenda sináptica monoamina oxidase (MAO) que faz o catabolismo das catecolaminas periférico e central (NORDAHL, SALO & LEAMON, 2003; ROTHMAN et al., 2001; BROWN, HANSON & FLECKENSTEIN, 2000).

Em alguns países as ANF e seus derivados ainda são utilizados na terapêutica médica, e também usados para fins não medicamentosos de forma ilícita (MACHALOVA et al., 2012). Em 2011, a exemplo de muitos outros países, foi proibida a comercialização bem como fabricação, pela Agência Nacional de Vigilância Sanitária – ANVISA no Brasil, dos medicamentos anfepramona, femproporex e mazindol, anfetamínicos supressores do apetite também chamados de anorexígenos (BRASIL - RDC No- 52/2011). Porém, há mais de 3 anos com a venda e prescrição proibidas no Brasil, as aminas anorexígenas voltam ao mercado farmacêutico e aos prescritores legalmente através da Resolução RDC n° 50/2014, em setembro de 2014, (BRASIL - RDC No- 50/2014) determinando a legalização da venda, sob prescrição em receituário de controle, das substâncias anfepramona, femproporex, mazindol e sibutramina, seus sais e isômeros. As aminas anorexígenas são derivados anfetamínicos e todas possuem efeitos psicoestimulantes característicos, possuindo efeitos de tolerância e até dependência semelhantes aos das ANF ilícitas.

O abuso de ANF vem tornando-se um problema global, principalmente através dos medicamentos. Estimativas mostram uma alta prevalência do consumo de psicoativos anfetamínicos entre motoristas profissionais de transportes de cargas nos últimos anos, que são usados por esses condutores para aumentar a sua atenção durante as viagens de longa distância (SOUZA et al., 2012; DAVEY, FREEMAN e LAVELLE, 2009).

Estima-se que 24,6 milhões de americanos com 12 anos ou mais de idade sejam usuários de drogas ilícitas, o que representa 9,4 por cento da população (SAMHSA, 2014). O abuso de

ANF e seus derivados desencadeia diversas consequências negativas, incluindo a dependência que é uma doença crônica, recidivante, caracterizada pela busca compulsiva pela droga acompanhada por alterações funcionais e moleculares no cérebro (NIDA, RESEARCH REPORT SERIES, 2013).

As drogas psicoestimulantes como a anfetamina e a cocaína também podem ser utilizadas em modelos para avaliar as alterações no sistema dopaminérgico e o desenvolvimento de adicção. Essas substâncias são utilizadas em modelos de preferência por drogas e autoadministração há muitos anos.

A autoadministração de drogas é um protocolo que avalia alguns parâmetros de adicção através da frequência de reforços que o animal executa através do acionamento de uma alavanca que ao ser ativada infunde a substância via endovenosa, esse protocolo tem contribuído muito para a compreensão e tratamento da adicção (PANLILIO & GOLDBERG, 2007).

Estudos recentes realizados por nosso grupo de pesquisa mostraram o desenvolvimento de preferência por anfetamina e de sintomas de ansiedade em ratos avaliados em protocolo de preferência condicionada por lugar (PCL) e labirinto em cruz elevado respectivamente (KUHN et al., 2013/2015), e exacerbação de outras anormalidades neuropsiquiátricas verificado no protocolo de mania (TREVIZOL et al., 2011/2013) após a suplementação com AGT durante uma ou duas gerações, o que reflete a sua influência e incorporação nas membranas neuronais. O protocolo de preferência condicionada de lugar (PCL) indica comportamento de busca pelos efeitos da droga através da associação com o ambiente, local onde o animal recebeu a droga, já o protocolo de autoadministração de drogas associa a busca com o comportamento de auto infusão da droga, assim torna-se de grande relevância investigar a influência desses ácidos graxos, bem como a modificação das membranas neuronais, nesses dois tipos de protocolos que podem indicar o potencial de busca pelos efeitos hedônicos de drogas psicoestimulantes como a anfetamina, que atua diretamente sobre o sistema dopaminérgico.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a influência de suplementações com diferentes óleos / gordura sobre parâmetros comportamentais associados à busca por anfetamina, determinar também a incorporação de diferentes ácidos graxos, marcadores de danos oxidativos e moleculares em áreas dopaminérgicas do cérebro de ratos.

2.1.1 Objetivos específicos

Protocolo experimental 1:

- Avaliar parâmetros de preferência à anfetamina através de preferência por lugar condicionado, bem como avaliar comportamentos de ansiedade e locomoção relacionados à abstinência da droga em animais de 2ª geração nascidos de matrizes suplementadas desde o período pré-concepcional com os diferentes ácidos graxos.
- Avaliar a incorporação de ácidos graxos e marcadores bioquímicos de estresse oxidativo em áreas cerebrais dopaminérgicas dos mesmos animais de 2ª geração expostos às diferentes suplementações de óleos / gordura;

Protocolo experimental 2:

- Avaliar alguns parâmetros de adicção através da autoadministração de anfetamina, bem como avaliar comportamentos de ansiedade e locomoção relacionados à abstinência da droga em animais de 1ª geração nascidos de matrizes suplementadas desde o período pré-concepcional com os diferentes ácidos graxos.
- Avaliar a incorporação de ácidos graxos e a expressão molecular de proteínas do BDNF, transportador de dopamina e receptores dopaminérgicos D1 e D2 em diferentes áreas cerebrais dos mesmos animais de 1ª geração expostos aos diferentes óleos / gorduras.

3 ARTIGOS CIENTÍFICOS

Uma parte dos estudos inseridos nesta tese apresenta-se sob a forma de artigo científico. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos, os quais estão dispostos da mesma forma em que foram publicados.

3.1 Artigo 1

TOXICOLOGICAL ASPECTS OF *TRANS* FAT CONSUMPTION OVER TWO SEQUENTIAL GENERATIONS OF RATS: OXIDATIVE DAMAGE AND PREFERENCE FOR AMPHETAMINE

Fábio T. Kuhn, Fabíola Trevizol, Verônica T. Dias, Raquel C. S. Barcelos, Camila S. Pase, Karine Roversi, Caren T. D. Antoniazzi, Katiane Roversi, Nardeli Boufleur, Dalila M. Benvegnú, Tatiana Emanuelli, Marilise E. Bürger

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Toxicological aspects of *trans* fat consumption over two sequential generations of rats: Oxidative damage and preference for amphetamine

Fábio Teixeira Kuhn [a], Fabíola Trevizol [a], Verônica Tironi Dias [a], Raquel Cristine Silva Barcelos [a], Camila Simonetti Pase [a], Karine Roversi [b], Caren Tatiane de David Antoniazzi [a], Katiane Roversi [b], Nardeli Boufleur [a], Dalila Moter Benvegnú [a], Tatiana Emanuelli [a,c], Marilise Escobar Bürger [a,b,*]

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HIGHLIGHTS

- Rats received omega-3/-6 and trans fat (TF) from gestation over two generations.
- Pups from 2nd generation were also supplemented until adolescence.
- Omega-6 and TF groups showed higher anxiety and amphetamine (AMPH)-preference.
- TF group showed impairments in the antioxidant defense system in cortex/hippocampus.
- Brain incorporation of trans/n-6 fatty acids may facilitate drug addiction.

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ABSTRACT

Chronic consumption of processed food causes structural changes in membrane phospholipids, affecting brain neurotransmission. Here we evaluated noxious influences of dietary fats over two generations of rats on amphetamine (AMPH)-conditioned place preference (CPP). Female rats received soybean oil (SO, rich in n-6 fatty acids (FA)), fish oil (FO, rich in n-3 FA) and hydrogenated vegetable fat (HVF, rich in trans fatty acids (TFA)) for two successive generations. Male pups from the 2nd generation were maintained on the same supplementation until 41 days of age, when they were conditioned with AMPH in CPP. While the FO group showed higher incorporation of n-3 polyunsaturated-FA (PUFA) in cortex/hippocampus, the HVF group showed TFA incorporation in these same brain areas. The SO and HVF groups showed AMPH-preference and anxiety-like symptoms during abstinence. Higher levels of protein carbonyl (PC) and lower levels of non-protein thiols (NPSH) were observed in cortex/hippocampus of the HVF group, indicating antioxidant defense system impairment. In contrast, the FO group showed no drug-preference and lower PC levels in cortex. Cortical PC was positively correlated with n-6/n-3 PUFA ratio, locomotion and anxiety-like behavior, and hippocampal PC was positively correlated with AMPH-preference, reinforcing connections between oxidative damage and AMPH-induced preference/abstinence behaviors. As brain incorporation of trans and n-6 PUFA modifies its physiological functions, it may facilitate drug addiction.

Abbreviations: AA, arachidonic acid; AMPH, amphetamine; CPP, conditioned place preference; DA, dopamine; DHA, docosahexaenoic acid; EFA, essential fatty acids; EPA, eicosapentaenoicacid; EPM, elevated plus maze; FA, fatty acids; FO, fish oil; HVF, hydrogenated vegetable fat; LA, linoleic acid; LC-PUFA, long chain polyunsaturated fatty acids; OF, open field; PUFA, polyunsaturated fatty acids; SO, soybean oil; TFA, trans fatty acids; PC, protein carbonyl; PND, post-natal day.

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1. Introduction

In recent decades, eating habits have been severely modified in different cultures around the world, especially among young people, with an increasing consumption of processed foods of higher palatability, which are rich in trans fatty acids (TFA), saturated fatty acids (SFA) and n-6 fatty acids (FA) (Mayneris-Perxachs et al., 2010; Baldini et al., 2009). From the toxicological point of view, chronic consumption of these fats may exacerbate imbalances between n-6/n-3 polyunsaturated fatty acids (PUFA), especially due to the decreased supply of n-3 FA (Ailhaud et al., 2006) that has occurred simultaneously. In this sense, humans have evolved on a diet in which the omega-6/omega-3 essential fatty acids (EFA) ratio was about 1, while in current Western diets this ratio is as low as 15/1–16.7/1, approximately (Simopoulos, 2002). Considering that the membrane lipid composition may be strongly influenced by the relative dietary abundance of polyunsaturated fatty acids (PUFA) (Hulbert et al., 2005; Walker et al., 2008), such change in eating habits may be reflected on neural membranes, whose key components are long-chain polyunsaturated fatty acids (LC-PUFA). In mammals, these membrane lipids are generated from dietary EFA, thus being incorporated into membrane phospholipids after desaturation and elongation.

Dietary provision of a balanced ratio of n-6 (linoleic acid; LA: 18:2n-6) and n-3 (α-linolenic acid; ALA: 18:3n-3) FA are crucial for the health of neonates (Hulbert, 2005; Walker et al., 2008 Walker et al., 2008). During the neonatal period (pregnancy and lactation), docosahexaenoic acid (DHA, 22:6 n-3), which is metabolite of ALA, exerts a fundamental role on cortical maturation, synaptogenesis and myelination, whose deficiency may be related to increased risk for cognitive deficits and mental disorders in adulthood. More precisely, DHA is incorporated into brain/retinal membranes, modulating their fluidity and permeability (Larqué et al., 2002; Hanebutt et al., 2008), while arachidonic acid (AA, 20:4 n-6), a metabolite of LA, is a bioactive precursor of eicosanoids such as prostaglandins and leukotrienes with pro-inflammatory activity (Vines et al., 2012; Santos et al., 2012; Tian et al., 2011). Interestingly, human studies have associated n-3 PUFA deficiencies with an increased vulnerability to affective disorders and aggression besides addiction to psychostimulant drugs (Buydens-Branchey et al., 2008, 2003a,b). In this sense, blood PUFA levels were found to be lower in relapsed than in non-relapse individuals, whose PUFA levels were satisfactory (Buydens-Branchey et al., 2003b).

Drug abuse is a major issue of public health at present. Amphetamine (AMPH) is a psychostimulant drug with high addictive potential, characterized by compulsion, craving and seeking for the drug, which persist despite serious adverse consequences. Its action occurs directly on the monoaminergic system, where it is able to inhibit dopamine (DA) reuptake, thus increasing cytosolic DA release (Sulzer, 2011). According to the National Survey on Drug Use and Health (NSDUH), approximately 1.2 million people (0.4% of the US population) were users of methamphetamine (METH) in 2012 and this number can be even greater if licit amphetamine derivatives such as methylphenidate are accounted. In fact, while methylphenidate has an approved medical use to treat attention-deficit hyperactivity disorder (ADHD) (NIDA, 2013), together with amphetamines it also has significant abuse liabilities, raising concerns about its non-medical use.

AMPH and its derivatives favor accumulation and auto-oxidation of DA in the synaptic cleft. Chronically, this cascade reaction generates DA quinones, which are closely related to oxidative insults (Perfeito et al., 2013; Carvalho et al., 2012; Sulzer, 2011), especially in mesocorticolimbic and hippocampal brain areas, where DA exerts a critical role.

A recent study by our group showed that trans fat consumption over one generation was able to modify addiction-related factors, as adolescent rats born under this dietary influence showed increased AMPH-preference (Kuhn et al., 2013). Indeed, repeated exposure to AMPH and/or cocaine has been experimentally used to evaluate addiction parameters in animal models, which include conditioned place preference (CPP). Widely used by other researchers (Taracha et al., 2014; Galaj et al., 2014) and ourselves (Antoniazzi et al., 2014; Segat et al., 2014), this behavioral paradigm allows to quantify rewarding symptoms related to psychostimulants in animals. Given the toxicological aspects of modern agriculture and the currently prevalent eating habits of the general population, we decided to give continuity to this line of research by further evaluating the influence of different fats consumed over two sequential generations of rats on AMPH-seeking behavior in adolescence.

2. Materials and methods

2.1. Animals and diets

Adult female Wistar rats (200–220 g) were placed in Plexiglas cages (two animals per cage) with free access to food and water in a room with controlled temperature (23 ± 1 °C) under a 12 h light/dark cycle with lights on at 7:00 a.m. Rats were randomly assigned to one of four experimental groups ($n = 8$ for each group): Control (C, received only water), soybean oil (SO, rich in n-6 FA), fish oil (FO, rich in n-3 FA) and HVF (rich in TFA), which were supplemented daily by oral gavage with 3.0 g/kg body weight from conception until weaning of pups. Supplementations were administered according to previous studies of our own (Trevizol et al., 2013; Pase et al., 2013; Kuhn et al., 2013) and of other groups (Vines et al., 2012; Ferraz et al., 2011). The different amounts of polyunsaturated, saturated, monounsaturated, omega-3, omega-6 and trans fatty acids present in the different supplementations are shown in Table 1. 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 throughout pregnancy and lactation until weaning of the litter of the second generation, when the male pups were also maintained on the same diet until 40 days of age (Fig. 1).

The body weight gain of dams and their offspring was monitored throughout the study period. The experimental protocol was approved by the Animal Ethical Committee (Universidade Federal de Santa Maria-UFSM-04/2012), which is affiliated to the Council for Control of Animal Experiments (CONCEA), following international norms of animal care and maintenance.

2.2. Drugs and solutions

DL-amphetamine (Merck, Germany) 4 mg/mL (Antoniazzi et al., 2014; Segat et al., 2014; Kuhn et al., 2013).

Table 1
Total percentage of fatty acids in the supplementation of different groups.

Fatty acids	SO group	FO group	HVF group
Σ Saturated	10.85	17.56	15.49
Σ Monounsaturated	33.84	36.31	43.27
Σ Trans	0.38	0.29	11.72
Σ Polyunsaturated	54.03	45.62	29.52
Σ Sat:Uns ratio	0.12	0.21	0.19
Σ n-6 PUFA	49.98	21.23	27.58
Σ n-3 PUFA	4.79	18.7	1.94
Σ n6:n3 ratio	10:1	1:1	14:1

Abbreviations: SO (Soybean oil); FO (Fish oil); HVF (Hydrogenated vegetable fat).

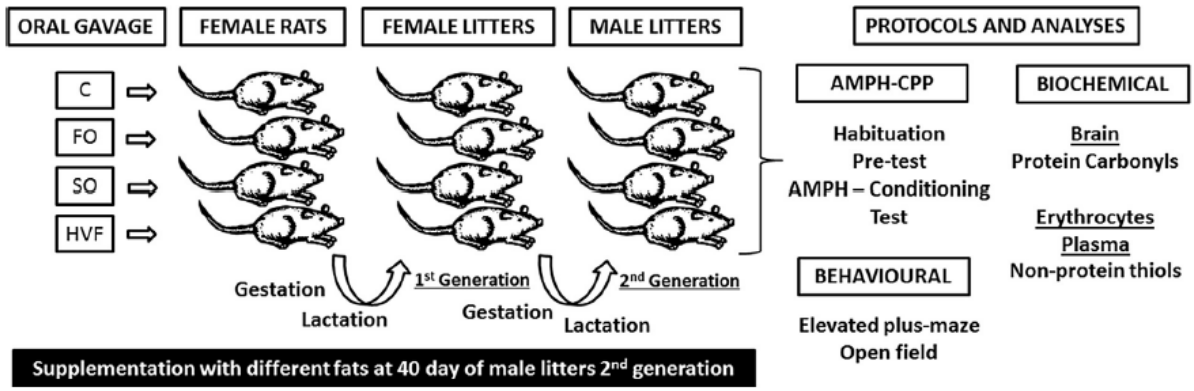

Fig. 1. Experimental design. Supplementations were conducted during pre-conception, pregnancy and lactation periods, which were maintained from weaning (PND 21) until PND40 over two sequential generations of male rats ($n = 8$). Abbreviations: PND: post-natal day; EPM: elevated plus-maze; CPP: conditioned place preference; AMPH: amphetamine; SAL: saline; PC: protein carbonyl; NPSH: Non-protein thiols.

2.3. Behavioral evaluations

2.3.1. Conditioned place preference (CPP) procedure

This experimental paradigm was performed as described by Thanos et al., 2010. The CPP apparatus had three compartments separated by manual guillotine doors: two boxes of equal size (45 × 45 × 50 cm) and equivalent intensity of light, but with different visual cues. One compartment had a smooth white floor and striped walls, while the other had a striped floor and smooth white walls. These two preference compartments were accessible by a central compartment (18 × 36 × 50 cm) which was gray with a smooth floor. The boxes were indirectly illuminated by incandescent light (40 W) of equal intensity at all times. The apparatus was cleaned with alcohol 20% using wet sponge and paper towel before the introduction of each animal. CPP was performed through the following steps: habituation, pre-test, conditioning and test. For habituation, animals were led to a room of behavioral evaluation, when the supplementations were stopped. At post-natal day (PND) 41 every animal was placed in both compartments (black and white) for 15 min so that it could settle down. The purpose of this procedure was to exclude exploratory behavior that is common in new environments during both the pretest and conditioning phases, thus avoiding misinterpretations. At PND 42, rats were placed in the middle of the neutral area and the time spent in each compartment was recorded for the following 15 min. Rats showing strong unconditioned aversion (less than 25% of the session time) or preference (more than 75% of the session time) for any compartment were discarded (Vazquez et al., 2006). Then, at PND 43, rats received AMPH (4 mg/kg ip) in one chamber, which was administered in alternation with saline at 4 h intervals. Immediately after each injection, animals were placed in opposite compartments of CPP, respectively, where they were allowed to access either chamber during each conditioning session of 25 min. This procedure was continued for eight consecutive days (PND 43–50), when AMPH was exclusively paired with a previously neutral environment whereas vehicle was exclusively paired with a second environment (Carlezon et al., 2002). On the CPP test day (PND 51), animals were not submitted to any invasive procedure, but were placed in the middle chamber with doors open for 15 min and given access to both chambers. The time spent in the drug-paired environment was interpreted as preference (Brenhouse and Andersen, 2008).

2.3.2. Elevated plus maze (EPM) test

At PND 52 anxiety-like symptoms related to drug abstinence were quantified in the EPM task. The apparatus consisted of a platform elevated 50 cm from the floor. Two opposite arms (50 × 10 cm) were enclosed by 40 cm high walls whereas the other two arms had no walls. The four arms had at their intersection a central platform (10 × 10 cm), which gave access to any of the four arms. The rats were placed individually in the central platform facing an open arm and observed for 5 min. In this test, rats display a variety of behaviors which are based on the innate fear rodents have for open and elevated spaces (Montgomery, 1955): while the time spent in the open arms is related to lower anxiety grade, behaviors of fear and risk are quantified by time spent in the closed arms (Rodgers et al., 1997; Hlavacova et al., 2010); the total number of entries in both open and closed arms was used as index of locomotor activity (Rodgers and Dalvi, 1997). The apparatus was cleaned with a 20% alcohol solution using wet sponge and paper towel before the introduction of each animal. Observers were blind to treatment during all behavioral observations.

2.3.3. Open-field (OF) task

At PND 53, locomotor and exploratory performances of the animals during drug-abstinence were quantified in the OF paradigm. This apparatus consisted of an arena (42 × 42 × 28 cm) divided into twelve quadrants (Kerr et al., 2005). The number of square crossings (horizontal squares crossed) and rearings (vertical movements) were recorded for 5 min and used as measures of spontaneous locomotor activity and exploratory behavior, respectively, which are primarily related to the locomotor index (Henderson et al., 2004). The OF was disinfected after each test.

2.4. Fatty acids profile of cortex and hippocampus

The fat was extracted from tissue samples using chloroform and methanol as described by Bligh and Dyer 1959. 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 H2SO4 solution (Hartman and Lago, 1973). Methylated fatty acids were analyzed using an Agilent Technologies gas chromatograph (HP 6890 N) equipped with a capillary column DB-23 60 m × 0.25 mm × 0.25 μm) and 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, USA and C 22:5n-6 from NuChek Prep. Inc. Elysian, MN, USA) were run under the same conditions and the

subsequent retention times were used to identify the FA. FA were expressed as percentage of the total FA content.

2.5. Biochemical measurements

Following 24 h from the last behavioral observation, animals were anesthetized with xylazine/ketamine (20 and 100 mg/kg, ip, respectively) and euthanized by exsanguinations. The collected blood (collected by cardiac puncture in heparinized tubes) was centrifuged at 1300 × g for 15 min for plasma and used for non-protein thiols (NPSH) assay. Brains were immediately removed and cut coronally at the caudal border of the olfactory tubercle. Cortex and hippocampus were dissected out and homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer (pH 7.4) for determination of protein carbonyl (PC) levels, which estimate oxidative damage to proteins, and NPSH levels, which estimate the antioxidant defenses.

2.5.1. Protein carbonyl (PC) quantification

PC was quantified by the method of Levine et al. (1994), with some modifications. Soluble protein was mixed with 2,4-dinitrophenylhydrazine (DNPH; 10 mMin 2 M HCl) or HCl (2 M) and incubated at room temperature for 1 h. Denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, with 3% sodium dodecyl sulfate), ethanol (99.8%) and hexane (99.5%) were added, mixed by shaking and centrifuged. The protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturing buffer. Each DNPH sample was read at 370 nm in a spectrophotometer against the corresponding HCl sample (blank). The results were expressed as nmol carbonyl/g tissue.

2.5.2. Non-protein thiols (NPSH)

Non-protein thiols (NPSH) content was determined after reaction with 5,5-dithiobis-(2-nitrobenzoic acid); the yellow color developed was read at 412 nm, in accordance with Boyne and Ellman, 1972. A standard curve using glutathione was constructed in order to calculate the content of NPSH, expressed as nmol NPSH/mL plasma.

2.6. Statistical analysis

Levene's test was applied in order to verify the homogeneity of the data. Differences between test and pretest in CPP were assessed by *t*-test paired samples. Behavioral and biochemical measurements were analyzed by one-way ANOVA followed by Duncan's multiple range test, when appropriate (Software package Statistica 8.0 for Windows was used). All of the data are expressed as means ± SEM. A *P*-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Body weight

No differences in body weight gain were observed across the experimental groups of dams and pups. At 40 days of age, the body weight of the second generation pups were 134.9 ± 1.01; 135.2 ± 1.09; 137.8 ± 0.52 and 139.33 ± 5.11, for control (C), soybean oil (SO), fish oil (FO) and hydrogenated vegetable fat (HVF), respectively, which are expressed as mean ± S.E.M.

3.2. Behavioral evaluations

3.2.1. The development of AMPH preference evaluated in CPP is shown in Fig. 2

While no significant differences of place preference between the different supplemented groups were observed during the pre-test (Fig. 2), paired *t*-test showed that C, SO ($P < 0.05$) and HVF ($P < 0.001$), but not FO, remained a longer time in the AMPH-conditioned compartment as compared to their respective pre-test values. One-way ANOVA [$F = 3.29$; $P < 0.001$] followed by Duncan's test of AMPH conditioning groups showed that SO and HVF displayed higher AMPH preference than both C and FO did, while the latter showed no preference for any compartment.

3.2.2. Anxiety-like symptoms evaluated in Elevated Plus Maze (EPM) are shown in Fig. 3

One-way ANOVA revealed a significant influence of supplementation on time spent in both open and closed arms [($F = 11.96$ and 9.18 and $P < 0.001$], respectively. Duncan's test showed that SO and HVF spent less time in the open arms (Fig. 3A) and more time in the closed arms (Fig. 3B) than both C and FO did, whose times spent in these compartments were comparable. In addition, no significant differences between the experimental groups were

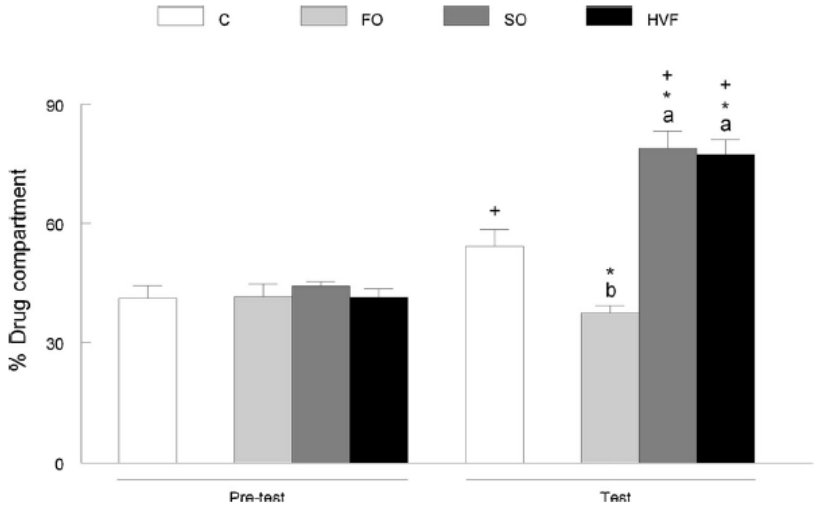

Fig. 2. Influence of different supplementations over 2nd generation of young rats on the development of AMPH preference. Animals were conditioned or not with AMPH (4 mg/kg/8 days) and evaluated in conditioned place preference (CPP). Data are expressed as mean ± S.E.M.; ($n = 8$). Different lowercase a,b indicates significant difference between supplementations (C, SO, FO and HVF) on test day. * Indicates significant difference between the supplemented groups (SO, FO and HVF) and control group (C) on test day. +indicates significant difference between test and pre-test (paired samples *t*-test, $P < 0.05$) in the same oral supplementation. Abbreviations: C: control; SO: soybean oil; FO: fish oil; HVF: hydrogenated vegetable fat; AMPH: amphetamine.

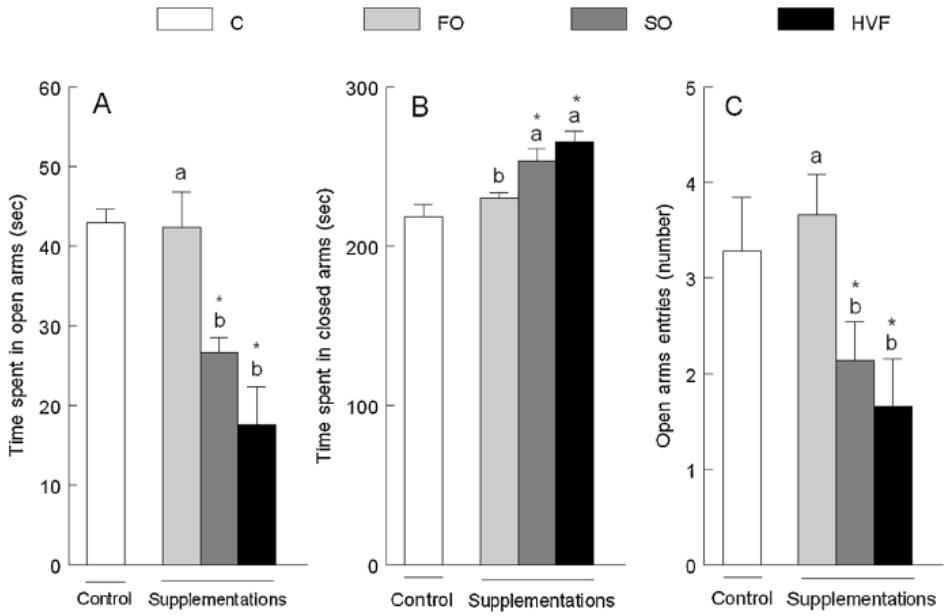

Fig. 3. Influence of different supplementations on anxiety-like symptoms of young rats evaluated in the elevated plus maze (EPM) task 48 h after the last AMPH injection. Rats were born of dams supplemented with different fat/oils from gestation/lactation and maintained in the same supplemented group until 40 days of age over two sequential generations, when they were conditioned with AMPH (4 mg/kg for 8 days). Data are expressed as mean ± S.E.M. ($n = 8$). Different lowercase a,b indicates significant difference between the experimental groups (C, SO, FO and HVF). * Indicates significant difference between each experimental group (SO, FO and HVF) and the control group (C). Abbreviations: C: control; SO: soybean oil; FO: fish oil; HVF: hydrogenated vegetable fat; AMPH: amphetamine.

observed in the total number of entries in both open and closed arms (Fig. 3C).

3.2.3. Locomotor activity and exploratory performance evaluated in open field (OP) are shown in Fig. 4

One-way ANOVA revealed a significant influence of the different supplementations on crossing number and rearing frequency [($F(3,28) = 6.59$, $P < 0.05$; 3.94, $P < 0.05$, respectively]. Post-hoc tests showed that FO supplementation decreased both crossing number and rearing frequency in relation to all other experimental groups, which showed comparable locomotor status (Fig. 4A and B).

3.3. Biochemical evaluations

3.3.1. Table 2 shows the influence of different fats on the FA incorporation in cortex and hippocampus of young rats from second generation

One-way ANOVA followed by Duncan's test showed that in relation to control, FO showed increased n-3 PUFA (18.8% and

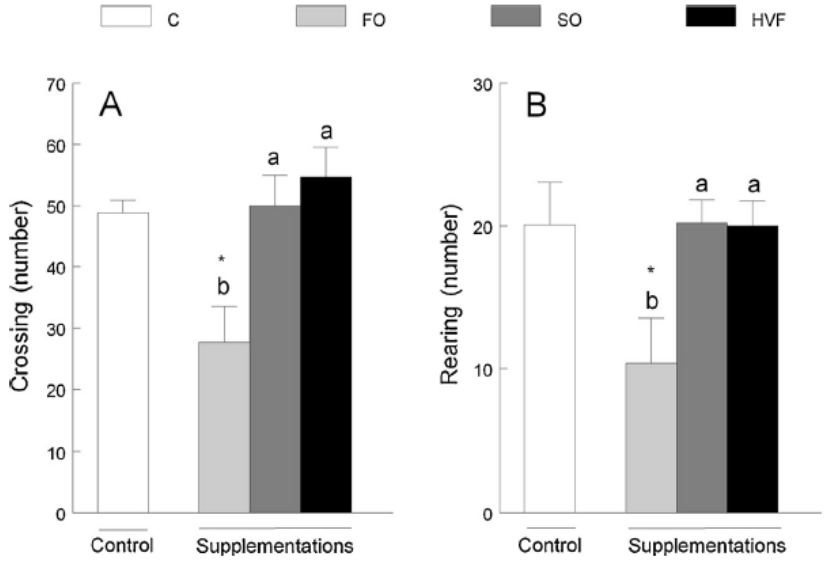

Fig. 4. Influence of different supplementations on locomotor status of young rats evaluated in the open-field (OF) task on PND 53. Rats were born of dams treated with different fat/oils from gestation/lactation and maintained in the same supplemented group until 40 days of age over two sequential generations, when they were conditioned with AMPH (4 mg/kg for 8 days). Data are expressed as mean ± S.E.M. ($n = 8$). Different lowercase a,b indicates significant difference between the experimental groups (C, SO, FO and HVF). * Indicates significant difference between each experimental group (SO, FO and HVF) and the control group (C). Abbreviations: C: control; SO: soybean oil; FO: fish oil; HVF: hydrogenated vegetable fat; AMPH: amphetamine.

Table 2
Fatty acids composition in hippocampus and cortex of rats supplemented with different oil/fat during two generations (% of total fatty acids identified).

Mean (±SEM)					
Tissue	Fatty acid	Control	Fish oil	Soybean oil	Hydrogenated vegetable fat
Cortex	Σ PUFA	27.50 ± 0.00	28.93 ± 0.08	27.60 ± 0.20	28.25 ± 0.21
	Σ n-3	11.75 ± 0.07[b]	13.96 ± 0.04[a]	11.62 ± 0.13[b]	11.91 ± 0.01[b]
	Σ n-6	15.88 ± 0,01[a]	14.71 ± 0.27[b]	15.57 ± 0.00[a]	15.89 ± 0.03[a]
	Σ trans FA	n.d.	n.d.	n.d.	0.13 ± 0.05
	n6/n3 ratio	1.36 ± 0.00[a]	1.07 ± 0.00[b]	1.33 ± 0.00[a]	1.37 ± 0.02[a]
Hippocampus	Σ PUFA	26.46 ± 0.24	27.14 ± 0.19	26.73 ± 0.52	26.84 ± 0.48
	Σ n-3	9.35 ± 0.11[b]	11.16 ± 0.09[a]	8.91 ± 0.15[b]	8.99 ± 0.16[b]
	Σ n-6	17.02 ± 0.01[b]	15.98 ± 0.17[c]	17.95 ± 0.03[a]	17.17 ± 0.03[b]
	Σ trans FA	n.d.	n.d.	n.d.	0.22 ± 0.13
	n6/n3 ratio	1.83 ± 0.02[a]	1.43 ± 0.01[b]	2.03 ± 0.02[a]	1.94 ± 0.04[a]

Different lowercase indicates significant difference between supplementations in the same tissue. One way ANOVA revealed a significant influence of the supplementations on cortex ($P < 0.05$) and hippocampus ($P < 0.01$).

19.35%), as well as decreased n-6 PUFA (7.36% and 6.11%) in cortex and hippocampus, respectively Table 2. These incorporations were also significantly higher for n-3 and lower for n-6 PUFA than those observed in SO and HVF. Overall, in comparison to C, FO showed decreased n-6/n-3 FA ratios (21.3 and 21.85%) in the same brain areas, respectively. Still, SO showed increased n-6 PUFA (5.46%) in hippocampus only. In contrast, HVF favored TFA incorporation by 0.13% and 0.22% in both cortex and hippocampus, respectively, something which was not observed in the other experimental groups (C, FO SO) Table 3.

3.3.2. Estimation of oxidative damage quantified by protein carbonyl (PC) levels in cortex and hippocampus is shown in Fig. 5

One-way ANOVA showed a significant influence of different supplementations on PC levels in cortex and hippocampus [$F(3,28) = 27.15$, $P < 0.001$; 5.45, $P < 0.05$, respectively]. Duncan's test showed that PC levels in cortex were higher in HVF than in all of the other experimental groups, while this oxidative marker was higher in SO than in both FO and C. In fact, C and FO showed similar PC levels in this brain area. In hippocampus, HVF was related to increased PC levels in relation to both SO and C, but not in relation to FO. In fact, FO and SO groups showed comparable hippocampal PC levels (Fig. 5A and B).

Interestingly, PC levels in cortex were positively correlated with n-6/n-3 PUFA ratio ($r^2 = 0.46$, $P = 0.026$) and crossing ($r^2 = 0.70$, $P = 0.0015$) in FO, as well as with time spent in closed arms ($r^2 = 0.40$, $P = 0.0083$) of EPM. Additionally, PC levels in hippocampus showed a small but significant positive correlation with AMPH-CPP ($r^2 = 0.19$, $P = 0.022$) besides a negative correlation with time spent in open arms of EPM ($r^2 = 0.19$, $P = 0.044$).

Table 3
Correlations between behavioral parameters and biochemical analysis performed in brain tissues of the 2nd generation of young rats exposed to amphetamine-conditioning.

Tissue	Parameters	Correlation	*P* value	
Cortex	Protein carbonyls	Crossing	(+) $r^2 = 0.70$	0.0015
		Rearing	(+) $r^2 = 0.37$	0.036
		OA entries (EPM)	(−) $r^2 = 0.31$	0.021
		Time spent in CA (EPM)	(+) $r^2 = 0.41$	0.083
		n3/n6 ratio	(−) $r^2 = 0.46$	0.026
Hippocampus	Protein carbonyls	AMPH-CPP	(+) $r^2 = 0.2$	0.022
		Time spent in OA (EPM)	(−) $r^2 = 0.19$	0.044

Abbreviations: EPM: elevated plus-maze; OA: open arms; CA: closed arms.

3.3.3. Estimation of antioxidant defenses determined by non-protein thiols (NPSH) levels in plasma and red blood cells (RBC) is shown in Fig. 6

One-way ANOVA showed a significant influence of the different supplementations on NPSH levels in plasma and RBC [$F(3,28) = 3.27$, $P < 0.05$; 3.07, $P < 0.05$, respectively.] HVF-supplemented animals showed decreased NPSH levels in both plasma and BRC as compared to C, FO and SO, whose levels were similar to each other Fig. 6.

4. Discussion

In our study, different supplementations during two sequential generations of rats, from six weeks before conception and through pregnancy and lactation, exerted a significant influence on drug preference in conditioned place preference (CPP). CPP is a well-established behavioral model that has been used to assess symptoms of reward and relapse to addictive drugs by several research groups (Taracha et al., 2014; Galaj et al., 2014; Thanos et al., 2010; Mathews et al., 2010; Segat et al., 2014; Kuhn et al., 2013; Antoniazzi et al., 2014). Here, SO- and HVF-supplemented rats showed a higher preference for the AMPH-conditioned compartment. Interestingly, these same experimental groups displayed a higher anxiety degree, as observed in the EPM. In contrast, the FO group showed no place preference, no anxiety-like symptoms and a decreased locomotor index, indicating a low index of emotionality. In line with our findings, a reduced emotionality was also previously observed in FO-supplemented rats exposed to stress, partially reinforcing our current findings (Ferraz et al., 2011).

Additionally, this study showed significant influences of the oils/fat on FA incorporation in some brain areas: (i) SO was related to increased n-6 PUFA incorporation in hippocampus; (ii) HVF allowed TFA incorporation in both cortex and hippocampus; (iii) FO showed increased n-3 and reduced n-6 PUFA incorporation in both cortex and hippocampus, thus reflecting on the n-6/n-3 PUFA ratio, as previously reported (Pase et al., 2013; Simopoulos, 2002, 1991). From these outcomes, it is possible to propose that different PUFA brain incorporations were able to affect the AMPH-preference observed in the CPP paradigm. In fact, HVF contains considerable amounts of TFA, which is replicated in the brain incorporation, especially in early life periods, thus affecting DA neurotransmission (Acar et at., 2003). More precisely, these authors showed that trans FA incorporation in the neuronal membranes of rats was related to increased DA levels, suggesting changes in gene expression and transcription of proteins involved in DA metabolism. Although we have not measured the levels of dopamine in the brain we hypothesized by the findings of previous study (Acar et al., 2003). Furthermore, brain incorporation of TFA

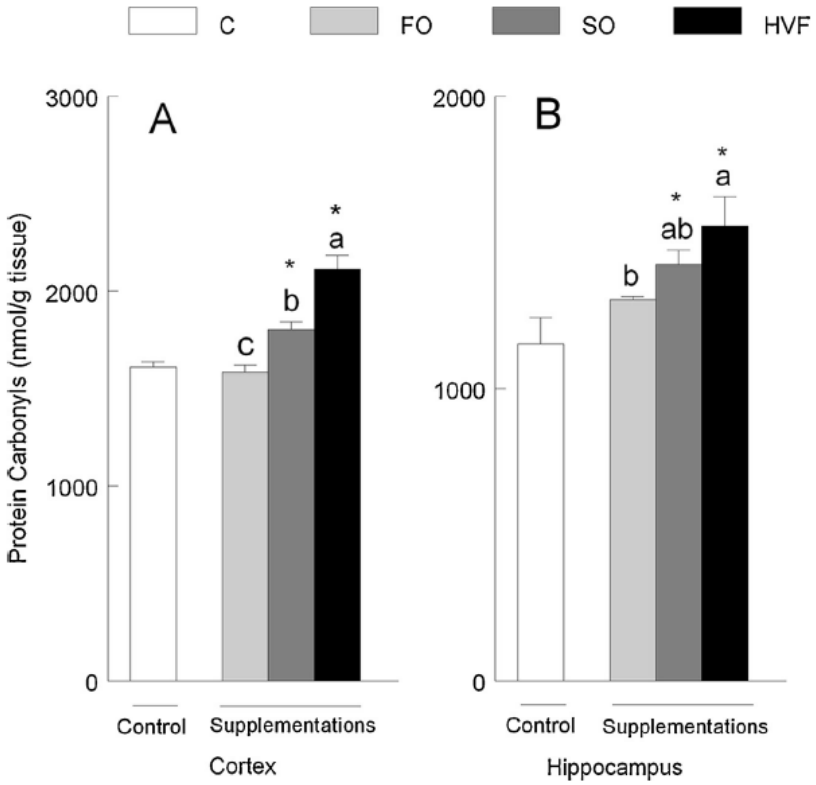

Fig. 5. Influence of SO, FO, or HVF supplementation on the protein carbonyl (PC) levels in cortex (Fig. 5A) and hippocampus (Fig. 5B) of young male rats born of dams and grandmothers supplemented with different fat/oils from gestation/lactation and maintained from weaning under the same supplementation. At 40 days of age, male rats were treated with AMPH (4 mg/kg for 8 days) and submitted to biochemical evaluations. Data are expressed as mean ± S.E.M. Different lowercase a,b,c indicates significant difference between experimental groups (C, SO, FO and HVF). * Indicates significant difference between each experimental group (SO, FO and HVF) and the control group (C). Abbreviations: C: control; SO: soybean oil; FO: fish oil; HVF: hydrogenated vegetable fat; AMPH: amphetamine.

also conferred greater rigidity to neuronal membranes (Alam et al., 1989).

Since SO is rich in n-6 FA, this supplementation promoted an increased incorporation of n-6 PUFA derivatives in the hippocampus, as also observed in first generation rats supplemented with SO (Trevizol et al., 2013). Indeed, a high intake of n-6 PUFA is able to increase their more pro-inflammatory derivatives (Simopoulos, 2002), contributing to additional damage to CNS, which may have influenced the AMPH-CPP and anxiety-like behavior observed in this experimental group.

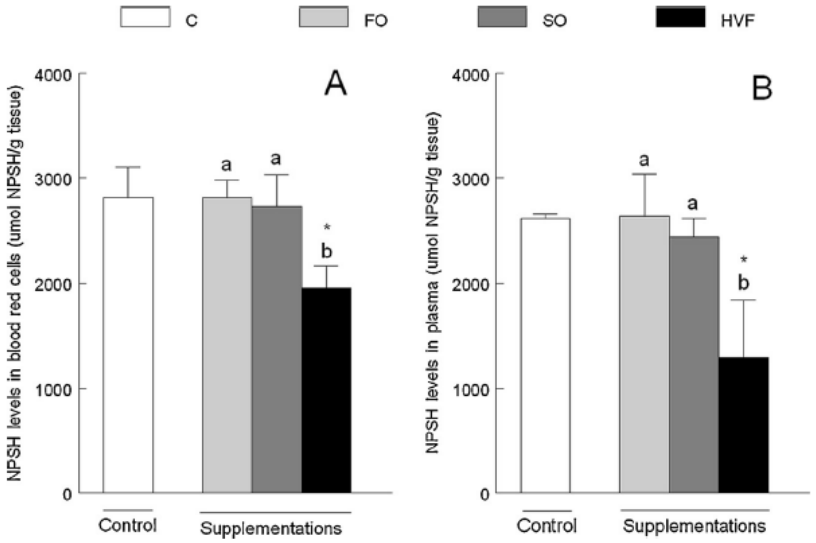

Fig. 6. Influence of different fats supplementation on non-protein thiols (NPSH) levels in blood of young rats. Animals were born of dams treated with different fats from gestation/lactation during two generations. Pups were maintained with the same original supplementation until 40 days of age, when they were treated with amphetamine (4 mg/kg) or its vehicle for 8 days. Data are expressed as mean ± S.E.M. Abbreviations: C: Control; SO: soybean oil; FO: fish oil; HVF-hydrogenated vegetable fat. Different lowercase a,b indicates significant difference between supplementations in the same tissue; * Indicates significant difference from control group.

On the other hand, it is possible to assume that FA incorporation in cortex and hippocampus in the FO group allowed higher stabilization of DA neurotransmission, as the presence of n-3 PUFA in neuronal phospholipid membranes is able to modify phospholipase A2 (Sarsilmaz et al., 2003), whose activity results in DHA and EPA generation. These eicosanoids exert regulatory functions in biochemical and physiological processes, influencing both fluidity and plasticity of the brain membranes. In more detail, DHA is a precursor of neuroprotectin D1, which provides anti-inflammatory and antiapoptotic properties to the neural membranes (Bazan, 2007).

So far, there are no published studies about the influence of different fats supplemented during two sequential generations of rats on AMPH-induced drug preference. A recent study of our group showed drug preference development in animals supplemented during the first generation (Kuhn et al., 2013). Of particular importance, Alsted and Hoy (1992) showed different brain FA incorporation across two generations of rats, indicating that the phospholipid composition of the neural membranes may be modified through generations. Apart from the preference for psychostimulant drugs, previous studies have shown additional behavioral impairments related to brain incorporation of n-6 PUFA and TFA, which were related to high anxiety scores and memory damage (Teixeira et al., 2011), movement disturbances in older rats (Teixeira et al., 2012) and increased susceptibility to develop mania-like symptoms (Trevizol et al., 2013). Moreover, HVF-supplemented rats showed agitation and higher vulnerability to stressful situations (Pase et al., 2013), indicating that trans fat- and, to a lesser extent, n-6 FA-rich diets during early life may favor the development of neuropsychiatric conditions in adolescence and/or adulthood. In fact, human studies have shown a relationship between aggressive behavior and lower n-3 PUFA levels in plasma and an almost significantly higher n-6/n-3 PUFA ratio (Buydens-Branchey et al., 2003a).

According to Passos et al. (2012) a long-term maternal diet with high saturated and monounsaturated FA content could affect key components of the midbrain dopaminergic system besides two distinct dopaminergic cell populations of *substantia nigra*, indicating that EFA restriction in multigenerational model is able to modify the tyrosine hydroxylase protein level in the ventral midbrain and the size of dopamine neurons.

Considering that AMPH-treatment is able to increase DA release, while TFA, and in minor proportion n-6 PUFA, may modify the dopaminergic neurotransmission (Acar et al., 2003), the generation of DA metabolites favors reactive species accumulation and oxidative insults to the neural system, thus affecting its physiologic function. Consistent with this, amphetamine compounds have been described by exerting neurotoxic events in rat brains (Alves et al., 2007), which may be exacerbated in hyperthermia (Dias da Silva et al., 2013). The neurotoxicity of AMPH and its derivatives (Carvalho et al., 2012) involves heat shock protein 27 (Ruscher et al., 2011) and seems to result from monoamines action and MAO activity (Carmo et al., 2003).

Our findings confirmed previous studies of our group, as SO and HVF supplementations were related to an increased level of protein carbonyl (PC) in both cortex and hippocampus. In addition, only HVF showed reduced levels of NPSH in both BRC and plasma. As free thiols are a substrate for antioxidant enzymes like GSH peroxidase (GPX), it has a fundamental role in regulating the redox state for cellular proliferation or differentiation (Cho et al., 2005; Burdon, 1995), thus affecting oxidative processes. Interestingly, the FO group showed no oxidative damage to proteins, confirming its less pro-inflammatory property, as already described. On the contrary, a trans fat-rich diet was related to increased production of proinflammatory cytokines and OS development (Han et al., 2002). The oxidative stress that gives rise to carbonyl groups generally causes loss of catalytic or structural function of proteins, reinforcing that the PC levels resulting from consumption of TFA along two generations exert deleterious effects on the cellular function (Levine et al., 1994), because these oxidative changes of proteins by reactive species may be involved in the progression of different brain disorders. Additionally, we found some positive correlations of PC levels in cortex with n-6/n-3 PUFA ratio and with the locomotor index. In hippocampus, there was a positive correlation between PC levels and time spent in the closed arms of EPM, as well as a negative correlation between PC levels and AMPH-CPP, emphasizing influences between AMPH-induced oxidative damage and preference behaviors related to addictive drugs.

Although at this time we do not wish to compare these with our previous findings, an interesting finding must be highlighted: while SO supplementation in only one generation of animals was not associated with preference for AMPH, anxious behavior and brain oxidative damage, the second generation of SO-supplemented animals did show these impairments. This indicates that very long-term consumption of n-6 FA-rich-food to detriment of a balanced n-6/n-3 FA intake may modify neural systems and affecting mesocorticolimbic reward pathways, facilitating the craving for addictive drugs.

5. Conclusion

In this study, we found that transgenerational consumption of trans fat as well as high amounts of omega-6 FA may favor AMPH-preference together with increased anxiety-like behaviors and motor restlessness. This more recent eating habit may also favor oxidative insults that may cause damage to neural circuitries, facilitating unbalanced dopamine neurotransmission and contributing to drug reward. This study serves to alert public health officials about the toxicological aspects of increased processed food consumption in Western societies. The excessive intake of trans and n-6 FA contributes to changes in the membrane phospholipids composition, thus affecting the dopaminergic neurotransmission, which is closely related to drug addiction.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2014.10.001.

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3.2 Artigo 2

CROSS-GENERATIONAL TRANS FAT CONSUMPTION FAVORS SELF-ADMINISTRATION OF AMPHETAMINE AND CHANGES MOLECULAR EXPRESSION OF BDNF, DAT AND D1/D2 RECEPTORS IN THE CORTEX AND HIPPOCAMPUS OF RATS

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ORIGINAL ARTICLE

Cross-Generational *trans* Fat Consumption Favors Self-Administration of Amphetamine and Changes Molecular Expressions of BDNF, DAT, and D1/D2 Receptors in the Cortex and Hippocampus of Rats

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Abstract Amphetamine (AMPH) is an addictive psychostimulant drug whose use has been related to neurotoxicity. Experimentally, AMPH increases anxiety-like symptoms, showing addictive properties. In the last decades, the growing consumption of processed foods has provided an excess of saturated and *trans* fats in detriment of essential fatty acids, which may modify the lipid profile of brain membranes, thus modifying its permeability and dopaminergic neurotransmission. Here, we assessed the influence of brain incorporation of different fatty acids (FA) on AMPH self-administration. Three groups of young male rats were orally supplemented from weaning with a mixture of soybean oil (SO, rich in *n*-6 FA) and fish oil (FO, rich in *n*-3 FA), hydrogenated vegetable fat (HVF, rich in *trans* fatty acids—TFA), or water (control group). These animals were born from dams that were supplemented with the same fat from pregnancy to lactation. Anxiety-like symptoms and locomotor index were assessed in elevated plus maze and open-field (OF), respectively, while brain molecular expressions of dopaminergic receptors, dopamine transporter (DAT), and BDNF were determined in the cortex and hippocampus. HVF increased the frequency of AMPH self-administration and was associated with reinforcement and withdrawal signs as observed by increased anxiety-like symptoms. Contrarily, SO/FO decreased these parameters. Increased BDNF protein together with decreased DAT expression was observed in the hippocampus of HVF group. Based on these findings, our study points to a harmful influence of *trans* fats on drug addiction and craving symptoms, whose mechanism may be related to changes in the dopaminergic neurotransmission.

Keywords Addiction · Self-administration · Amphetamine · Omega-3 · *trans* fat · Dopamine

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Introduction

The abuse of psychostimulant drugs is a serious public health issue worldwide due to severe addiction and neuropsychiatric complications. The number of new users of amphetamines, mainly methamphetamine, among people aged 12 years old or older was 144,000 in 2013 (SAMHSA 2014). Nonmedical use of psychostimulant prescriptions and over-the-counter medicines remain a significant part of the teen drug problem. In the same year, 15 % of high-school teenagers used a prescription drug for nonmedical purposes. The survey shows continued abuse of DL-amphetamine, which is frequently used to treat attention deficit hyperactivity disorder (ADHD), with 7.4 % of teenagers reporting taking it for nonmedical reasons only in the past year, and 2.3 % reporting the abuse of Ritalin® (methylphenidate), another ADHD medication (NIDA, youth trends 2014). It is estimated that 24.6 million Americans are users of illicit drugs, what represents 9.4 % of the population (SAMHSA 2014).

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Long-term abuse of amphetamine (AMPH) derivatives has several negative consequences, including addiction, which is a chronic, relapsing disease, characterized by compulsive drug seeking, and their use is accompanied by functional and molecular changes in the brain (NIDA 2014). Studies have shown that brain-derived neurotrophic factor (BDNF) in mesocorticolimbic dopaminergic reward areas constitutes a modulator associated with psychostimulant drugs, also exerting significant influence on the structural and behavioral plasticity related to addiction (Hyman et al. 1991; Meng et al. 2013). Furthermore, BDNF modulates several behaviors related to addictive drugs such as amphetamine, cocaine, and nicotine (Shen et al. 2014; Lang et al. 2007; Gomez-Pinilla 2008; Tyagi et al. 2015), since its up-regulation has been observed in addiction. Indeed, BDNF exerts an essential role in establishing the proper number of dopaminergic neurons in the Central Nervous System (CNS) (Bousquet et al. 2009; Baquet et al. 2005).

Different experimental protocols have been used to assess rewarding effects of addictive drugs, including conditioned place preference (CPP), which assesses preference for the place where the animal received the drug (Panlilio and Goldberg 2007), and the self-administration paradigm. In fact, this experimental procedure is also able to estimate reinforcement through the increased frequency of drug administration. Taken together, these two animal models have been useful tools to understand pathophysiological parameters associated to drug addiction. Literature data have shown the involvement of D1 and D2 dopamine receptors in rewarding effects caused by psychostimulant drugs. More precisely, D2 receptors mediate reinforcement symptoms, whereas D1 play a permissive role by facilitating the development of addiction (Phillips et al. 1994; Maldonado et al. 1993; Beninger et al. 1989). In this sense, at least five distinct G protein-coupled receptor subtypes mediate diverse physiological actions of dopamine. Two D1-like receptor subtypes (D1 and D5) are coupled with G protein, activating the adenylyl cyclase. Other receptor subtypes belong to the D2-like subfamily (D2, D3, and D4), whose activation is related to inhibition of the adenylyl cyclase cascade (Missale et al. 1998). Moreover, the dopamine transporter (DAT) is located in pre-synaptic dopaminergic nerve terminals and regulates synaptic neurotransmission by controlling DA concentrations available for binding to pre-synaptic and post-synaptic DA receptors (Amara and Kuhar 1993). Since the DAT is anchored in the lipid bilayer, it could be expected that modifications in the architecture of neuronal cell membranes could alter its function, as already shown for membrane-associated proteins (Bourre et al. 1989; Kodas et al. 2002). Recent studies by our research group have shown the development of AMPH-preference and anxiety-like symptoms (Kuhn et al. 2013, 2015) besides other neuropsychiatric conditions (Trevizol et al. 2011, 2013, 2014) observed in rats of first and second generation supplemented with *trans* fats, thus reflecting their influence through the incorporation in neuronal membranes and the exacerbation of AMPH-induced damage.

Processed foods are rich in *trans* fatty acid (TFA), and their consumption can represent an incorporation of *trans*-derivatives in neural membranes, as already shown (Kuhn et al. 2015; Trevizol et al. 2011, 2013, 2014). Contrarily, incorporation of *n*-3/*n*-6 PUFA in membrane phospholipids is fundamental to maintain membrane integrity, thus preserving brain physiologic functions (Wandall 2008; Acar et al. 2003; Trevizol et al. 2011; Teixeira et al. 2011, 2012). Experimental studies have suggested that the highest incorporation of PUFA derivatives occurs during the perinatal period (Kuhn et al. 2013, 2015; Pase et al. 2013), which extends from pregnancy until the end of the breastfeeding period. Indeed, pregnancy is a crucial stage, as large amounts of *n*-6 and *n*-3 FA are needed for synthesis of phospholipids in order to complete fetal brain development and its neurogenesis. The passage of *n*-6 and *n*-3 PUFA across the fetal blood–brain barrier depends on relative amounts of FA in the blood of the fetus, further indicating the need for a balanced maternal diet (Chen et al. 2008; Green et al. 2008). Of particular importance, while a higher incorporation of *n*-3 PUFA has been related to the increased fluidity of neuronal membranes and the balanced density and activity of receptors (Yehuda et al. 2005), the presence of TFA in these membranes has been associated with memory loss, anxiety-like symptoms (Teixeira et al. 2011), and movement disorders (Teixeira et al. 2012).

Lambert (2006) has reported that natural and synthetic TFA have different structures and functions, and distinct effects on blood lipids: natural TFA are beneficial to health, whereas synthetic TFA are not. A recent United Nations high-level meeting has emphasized the importance of policies to reduce and/or eliminate TFA in the food supply (Beaglehole et al. 2011). The United States Food and Drug Administration (FDA) also released a proposal to limit the level of TFA in foods by considering partially hydrogenated oils not "generally recognized as safe" for use in food. In fact, some individuals might consume amounts that exceed the daily recommendation of <1 % of total energy from TFA (Arcand et al. 2014).

Based on this, it is important to study the influence of dietary fatty acid profile and the consequent repercussions on neuronal membranes, on reward behaviors, also assessing molecular markers related to the dopaminergic system and neuronal growth factors.

Methods

Animals and Experimental Procedure

Fifteen adult female Wistar rats (200–220 g) were placed in Plexiglas cages (one per cage) with free access to food and water in a room with controlled temperature (23 ± 1 °C) on a 12 h light/dark cycle with lights on at 7:00 am. Animals were supplemented by gavage (3 g/kg) once a day with soybean oil/fish oil (SO/FO), which contains an ideal ratio of *n*-6/*n*-3 (1:1) fatty acids, hydrogenated vegetable fat (HVF), which contains significant amounts of *trans* fat, while an additional control group (C) received water, as a control for possible behavioral changes related to the gavage procedure. The quantification of fatty acids present in each supplementation was determined by gas chromatography (Hartman and Lago 1973) (Table 1).

The rationale for oral administration of these different fats during development and growth is their incorporation into neuronal membrane phospholipids and possible influence on physiological brain functions (Jump 2002; Yehuda et al. 2002), which might influence addictive behaviors. In this sense, animals received the different fats by gavage, thus mimicking dietary fat intake. The chosen doses were based on pilot studies developed by our group as well as by other laboratories (Kuhn et al. 2015; Trevizol et al. 2013; Kuhn et al. 2013; Pase et al. 2013, Ferraz et al. 2011; Vines et al. 2012), which investigated incorporation of different FAs and their derivatives in brain tissues. Supplementations were initiated 2 weeks before mating, continued during pregnancy, which was confirmed by the presence of sperm in the vaginal smear, and maintained until post-natal day 21 (PND 21), when litters were weaned. In order to exclude genetic similarities, only one male pup of each litter was included in each experimental group ($n = 5$) after weaning. From weaning (post-natal day-PND 21) until PND 50, pups were maintained under the same supplementation as their dams, not by breastfeeding, but by gavage, similarly to their progenitors (Fig. 1).

Table 1 Total percentage of fatty acids in the supplementation of different groups

Fatty acids	SO/FO group	HVF group
Σ Saturated	26.91	33.58
Σ Monounsaturated	25.68	53.77
Σ *trans*	0.16	13.43
Σ Polyunsaturated	47.4	12.65
Σ Sat:uns ratio	0.37	0.51
Σ *n*-6 PUFA	23.74	12.29
Σ *n*-3 PUFA	23.58	0.36
Σ *n*6:*n*3 ratio	1.01	34.02

SO soybean oil, *FO* fish oil, *HVF* hydrogenated vegetable fat

The body weight gain of dams and their offspring was monitored throughout the experimental period. The experimental protocol was approved by the Animal Ethics Committee (Universidade Federal de Santa Maria-UFSM-04/2012), which is affiliated to the Council for the Control of Animal Experimentations (CONCEA), following international norms of animal care and maintenance.

Apparatus

For amphetamine self-administration, the animals were individually put in experimental Plexiglas chambers (30 × 30.5 × 24.5 cm^3), enclosed in light and sound attenuating boxes. The floor of the chambers consisted of a Plexiglas tray covered with sawdust. A hole in the ceiling allowed the passage and free movement of the tethered catheter (Instech Solomon® Winsum, The Netherlands), connected to a counterbalanced swivel and an infusion pump (Insight Equipments®, Ribeirão Preto, SP, Brazil). The front wall of the chamber contained one interchangeable panel. The panel was equipped with two levers, located 5 cm from the floor, two cue lights (red and green) above each lever, and a session light in the middle of the panel (12 cm from the floor).

Drugs and Solutions

DL-amphetamine (Merck, Germany) 0.025 mg/Kg/0.1 mL was adapted from Shahbazi et al. (2008).

Training

Training consisted of three 60-min sessions in fixed ratio schedule of reinforcement (FR), in which each response on the active lever (alternated between left and right sides) was reinforced with the delivery of 0.2 mL sucrose (6 %), followed by a 10-s time-out. Each rat was allowed continuous access to the sucrose solution throughout the session. Responding on the inactive lever had no scheduled consequence (Leão et al. 2013).

Surgery

24 h after the last training session, rats were implanted with permanently indwelling catheters (Instech Solomon® silicon tubing, inner diameter = 0.63 mm, outer diameter = 1.17 mm) into the right jugular vein under a combination of thiopental (25.0 mg/kg) and xylazine (15.0 mg/kg) anesthesia. The catheter was passed subcutaneously to the rat's back where it exited through a small incision and was affixed to a plastic pedestal mounted

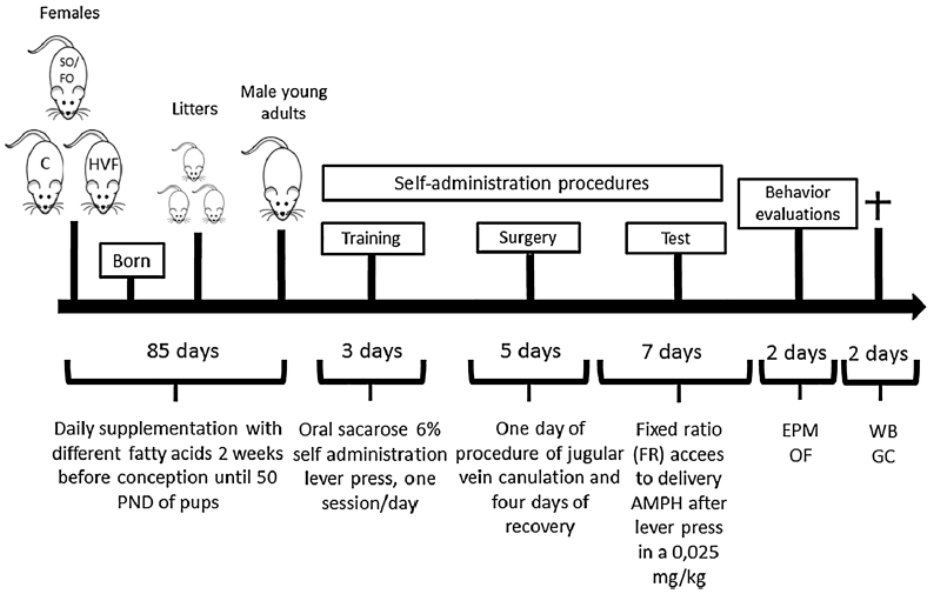

Fig. 1 Experimental design. Supplementations were conducted during pre-conception, pregnancy, and lactation periods, and were maintained from weaning (PND 21) until PND 50 over one generation of male rats ($n = 5$). *PND* post-natal day, *EPM* elevated plus maze, *OF* open field, *WB* western blotting, *GC* gas chromatography

inside a harness system (Instech Solomon®). Rats were allowed to recover from surgery for 5 days in their home cage with free access to food and water. They received cetoprophen 1 % (5.0 mg/kg; i.m.), and cefazolin (10.0 mg/kg; i.v.) for three consecutive days following surgery to prevent inflammation and infection. The catheter was flushed daily with heparinized saline (20 IU/mL) and 0.2 mL of saline in order to maintain its patency.

Acquisition of Amphetamine Self-Administration on FR

After a 5-day postsurgical recovery, the testing of spontaneous acquisition of amphetamine self-administration began. Sessions lasted 1 h and were performed daily for seven consecutive days during the light phase of the light/dark cycle. One priming-dose was injected into all animals in the beginning of the first two sessions, and these two priming-doses were not accounted. Sessions began when the two levers extended into the chamber. Noncontingent drug infusions were not administered. Lever-pressing was reinforced by IV injection of 0.025 mg/kg/0.1-mL amphetamine infusion in an FR. Behavioral assessments were conducted 24 h after the last exposure to amphetamine. The concentration of amphetamine solution was titrated daily for all subjects to adjust for body weight.

Elevated Plus Maze (EPM)

The apparatus consisted of a platform elevated 50 cm from the floor. Two opposite arms (50 × 10 cm^2) were enclosed by 40 cm-high walls, whereas the two other arms did not have walls. At their intersection, a central platform (10 × 10 cm^2) gave access to any of the four arms. On PND 66, rats were individually placed in the central platform facing an open arm, and observed in EPM for 5 min. In this test, rats display a variety of behaviors based on the innate fear rodents have for open and elevated spaces (Montgomery 1955): while time spent in the open arms is related to lower anxiety levels, behaviors of fear and risk are quantified by time spent in the closed arms (Rodgers and Dalvi 1997; Hlavacova et al. 2010). The total number of entries in both open and closed arms of EPM indicates that there is no locomotor artifact, whose observation is made only to assess impaired exploratory behavior (Cohen et al. 2012). The apparatus was cleaned with a 20 % alcohol solution using a wet sponge and paper towel before the introduction of each animal. Observers were blind to treatment during all behavioral observations.

Open-Field (OF) Test

On PND 67, animals were placed individually at the center of a circular open-field arena (50 cm of diameter) enclosed by matte white walls and a white floor divided into squares, adapted from Kerr et al. (2005). The OF was disinfected after each test (5 % alcohol solution). Crossing (indicative of locomotion) and rearing (indicative of exploration) numbers were quantified in conjunction, which are primarily related to the locomotor index (Henderson et al. 2004).

Tissue Preparations

One day after the last behavioral assessment, all animals were anesthetized (sodium thiopental, 50 mg/kg body

weight ip) and euthanized by cervical decapitation. Brains were removed and cut coronally at the caudal border of the olfactory tubercle to remove the cortex and hippocampus (Paxinos and Watson 2007), which were separated into two parts used for biochemical assays: fatty acids determination and molecular analysis. The rationale for selecting these brain structures is due to the fact that the hippocampus is the main brain area involved in the connection between environment in which drug is taken and reward circuits, making it a potential target for studying drug addiction. Furthermore, the cortex plays a critical role in reward circuits, modulated by dopamine. Together, these brain areas coordinate motivated behaviors by neurochemical changes in mesocorticolimbic pathways, which are induced by addictive drugs (Feltstein and See 2008; Hyman et al. 2006).

Fatty Acids Profile of Cortex and Hippocampus

Fat was extracted from tissue samples using chloroform and methanol as described by Bligh and Dyer 1959. 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 (Hartman and Lago 1973). Methylated fatty acids were analyzed using a gas chromatograph by Agilent Technologies (HP 6890N) equipped with a capillary column DB-23 60 m × 0.25 mm × 0.25 µm) and flame ionization detector. 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), oven temperature was held at 160 °C for 1 min, then 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 22:5*n*3 and PUFA no. 2 from Sigma, Saint Louis, MO, USA, and C 22:5*n*-6 from NuChek Prep. Inc., Elysian, MN, USA) were run under the same conditions, and subsequent retention times were used to identify the FA. FA was expressed as percentage of total FA content.

Immunoblotting

Cortical and hippocampal tissues were homogenized with a lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1 % NP40, 10 % glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 µg mL^{-1} aprotinin, 0.1 mM benzethonium chloride, and 0.5 mM sodium vanadate. Homogenates were then centrifuged, supernatants were collected, and total protein concentration was determined according to the MicroBCA procedure (Pierce, IL, USA), using bovine serum albumin (BSA) as standard. Briefly, protein samples were separated by electrophoresis on a 10 and 12.5 % polyacrylamide gel (according to protein molecular weight), and electrotransferred to a PVDF membrane (Millipore, MA, USA). Non-specific binding sites were blocked in Tris-buffered saline (TBS), pH 7.6, containing 5 % non-fat dry milk. Membranes were rinsed in buffer (0.05 % Tween-20 in TBS) and then incubated with primary antibodies: anti-actin (1:2000), anti-BDNF (1:300) anti-D1R (1:500), anti-D2R (1:500), anti-DAT (1:500) (Santa Cruz Biotechnology, Santa, Cruz, CA, USA) followed by anti-rabbit or anti-goat IgG horseradish peroxidase conjugate (1:40.000; Santa Cruz Biotechnology). After being rinsed with buffer, the immune complexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., NJ, USA) according to the manufacturer's instructions. The film signals were digitally scanned, and then quantified using ImageJ software. Actin was used as an internal control for Western blot such that data were standardized according to actin values.

Statistical Analysis

Data from self-administration of amphetamine were analyzed by two-way ANOVA (3 supplementations (C; SO/FO; HVF) × 7 periods (7 consecutive days). This last factor was considered a repeated measure and used to compare the number of lever press at different time points, followed by Duncan's multiple range test for all comparisons, when appropriate. Behavioral assessments from EPM and OF as well as molecular measurements were analyzed by one-way ANOVA followed by Duncan's multiple range test. A $P < 0.05$ value was regarded as statistically significant for all comparisons made, whose results were expressed as mean ± S.E.M.

Results

Body Weight

No differences in body weight gain were observed with experimental groups of dams and pups. At 50 days of age, the body weight (expressed as mean ± S.E.M.) of pups from first generation were 151.8 ± 4.3, 157.3 ± 1.4, 159.1 ± 2 for control (C), soybean oil/fish oil (SO/FO), and hydrogenated vegetable fat (HVF), respectively.

Influence of the Different Supplementations on Amphetamine (AMPH) self-Administration is Shown in Fig. 2

Two-way ANOVA of lever press number revealed a significant main effect of supplementation [$F(2,12) = 140.59$,

$P < 0.000$], a main effect of period [$F(6,72) = 118.01$, $P < 0.000$] and a significant supplementation × period interaction [$F(12,72) = 118.01$, $P < 0.000$].

No differences in lever press number were observed among experimental groups on the first day of AMPH self-administration. On the second day of AMPH exposure, HVF group was more active to press the lever than SO/FO group, although it was comparable to control. On the third day of AMPH self-administration, HVF showed a higher number of lever press in relation to both SO/FO and control groups, whose active behaviors were similar. From day 4 until day 7, HVF-supplemented group showed a higher number of lever press than both SO/FO and control group. In fact, SO/FO showed decreased AMPH self-administration in relation to control group.

Anxiety-Like Symptoms Assessed in Elevated Plus Maze (EPM) are shown in Fig. 3

One-way ANOVA revealed a significant influence of supplementation on time spent in both open and closed arms [$F(2,12) = 148.21$ and 14.70 and $P < 0.001$, respectively]. Post-hoc test showed that SO/FO group spent more time in the open arms (Fig. 3a) and less time in the closed arms of EPM (Fig. 3b), whereas HVF spent less time in the open arms (Fig. 3a) than SO/FO group. In fact, both C and HVF group spent comparable time in the closed arms (Fig. 3b). In addition, no significant differences were observed among experimental groups regarding total number of entries in both open and closed arms (Fig. 3c).

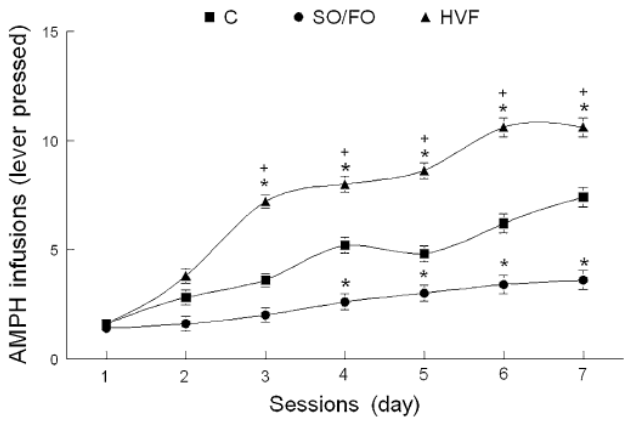

Fig. 2 Number of AMPH infusions (0.025 mg/Kg) in the self-administration protocol, after supplementation with different fatty acids, mixture of FO plus SO, HVF, and control during one generation. Data are expressed as mean ± S.E.M. ($n = 5$). *Asterisk* indicates significant difference from control (C group), *plus* indicates significant difference from SO/FO; (ANOVA followed by Duncan's test; $P < 0.05$). *C* control, *SO* soybean oil, *FO* fish oil, *HVF* hydrogenated vegetable fat, *AMPH* amphetamine

Locomotor Activity and Exploratory Performance Assessed in Open Field (OP) are Shown in Fig. 4

A significant influence (one-way ANOVA) of the different supplementations was evidenced on crossing number and rearing frequency [($F(2,12) = 23.49$, $P < 0.001$ and 3.42, $P < 0.05$, respectively]. Post-hoc test showed decreased crossing number (Fig. 4a) and rearing frequency (Fig. 4b) in SO/FO in comparison to both C and HVF groups. While HVF group showed increased crossing in relation to C group, these two experimental groups showed comparable rearing frequency (Fig. 4a ,b).

Influence of Different Supplementations on Brain-Derived Neurotrophic Factor (BDNF) and Dopamine Transporter (DAT) Levels (Fig. 5) in the Cortex

One-way ANOVA revealed a significant influence of the different supplementations on BDNF and DAT levels [$F(2,12) = 6.67$, and 8.90, $P < 0.05$, respectively] in the brain cortex. While SO/FO group showed decreased BDNF and increased DAT levels in comparison to both C and HVF groups, HVF supplementation showed an inverse influence, since a tendency of higher BDNF and lower DAT was observed in the cortex of HVF group, whose values were significantly different from SO/FO group (Fig. 5a, b).

Influence of Different Supplementations on Brain-Derived Neurotrophic Factor (BDNF) and Dopamine Transporter (DAT) Levels (Fig. 5) in the Hippocampus

Analyses (one-way ANOVA) show a significant influence of the different supplementations on BDNF and DAT levels [$F(2,12) = 7.17$, and 9.54, $P < 0.05$, respectively] in the hippocampus. While BDNF expression was increased in HVF and decreased in SO/FO group, DAT level was increased in SO/FO and decreased in HVF group, being their expression significantly different from one another as well as from C group (Fig. 5c ,d, respectively).

Influence of Different Supplementations on Dopamine D1 Receptor (D1R) and Dopamine D2 Receptor (D2R) Levels in the Cortex and the Hippocampus (Fig. 6)

One-way ANOVA revealed a significant influence of the different supplementations on D1R and D2R levels [$F(2,12) = 15.70$, $P < 0.05$ and 68.03, $P < 0.001$, respectively] in the cortex and [$F(2,12) = 12.82$, $P < 0.05$ and 47.76, $P < 0.001$, respectively] in the hippocampus. In the cortex, D1R decreased in SO/FO and increased in HVF

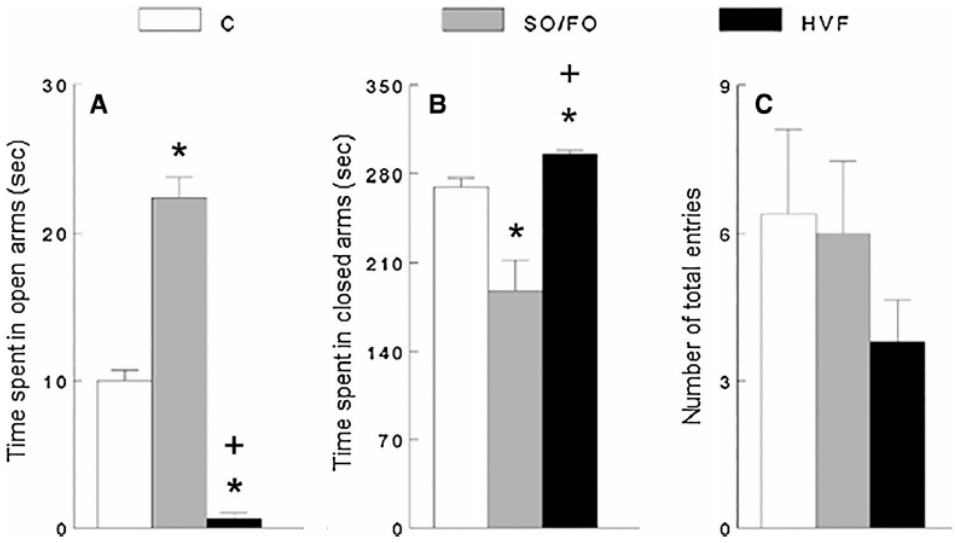

Fig. 3 Influence of different supplementations on anxiety-like symptoms of young pups, assessed in the elevated plus maze (EPM) 24 h after the last AMPH infusion. Rats were born of dams treated with different fats from gestation/lactation and maintained in the same supplemented group until 50 days of age. Data are expressed as mean ± S.E.M. ($n = 5$). *Asterisk* indicates significant difference from control (C group), *plus* indicates significant difference from SO/FO; (ANOVA followed by Duncan's test; $P < 0.05$). *C* control, *SO* soybean oil, *FO* fish oil, *HVF* hydrogenated vegetable fat, *AMPH* amphetamine

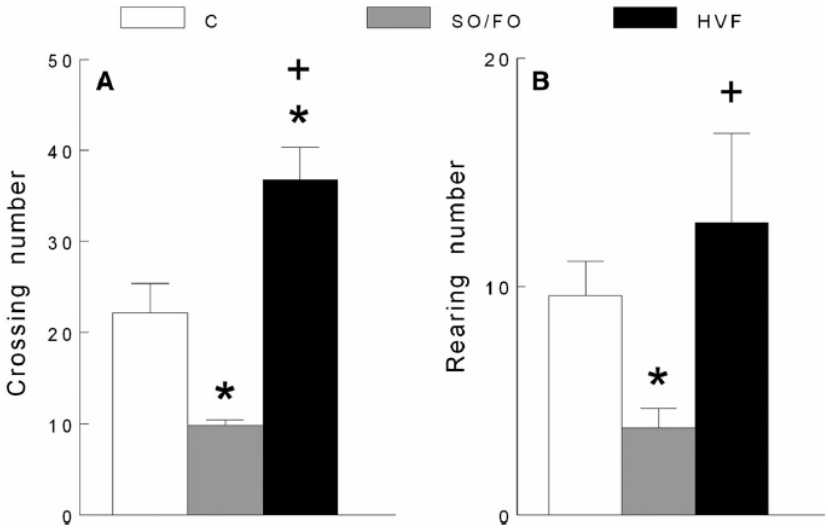

Fig. 4 Influence of different supplementations on locomotor status of young pups, assessed in open-field (OF) task. Animals were born of dams supplemented with the same fat from gestation/lactation and maintained in the same supplemented group from weaning until 50 days of age. Data are expressed as mean ± S.E.M. ($n = 5$). *Asterisk* indicates significant difference from control (C group), *plus* indicates significant difference from SO/FO; (ANOVA followed by Duncan's test; $P < 0.05$). *C* control, *SO* soybean oil, *FO* fish oil, *HVF* hydrogenated vegetable fat, *AMPH* amphetamine

group (Fig. 6a), being their expression different from one another and from C group, whereas D2R decreased in HVF group (Fig. 6b). In fact, D2R was comparable between C and SO/FO groups. In the hippocampus, D1R expression decreased (Fig. 6c) whereas D2R increased (Fig. 6d) in both SO/FO and HVF. Indeed, hippocampal D1R level was similar between SO/FO and HVF groups, while significant differences in D2R expression were observed between these two experimental groups.

Table 2 shows Influence of Different Fats on FA Incorporation in the Cortex and Hippocampus of Young Rats from First Generation

One-way ANOVA followed by Duncan's test showed that SO/FO showed decreased *n*-6/*n*-3 PUFA ratio (19.86 and 22 %, respectively) in the cortex in relation to both control and HVF groups. Furthermore, HVF group showed higher *n*-6/*n*-3 PUFA ratio in the hippocampus, besides an

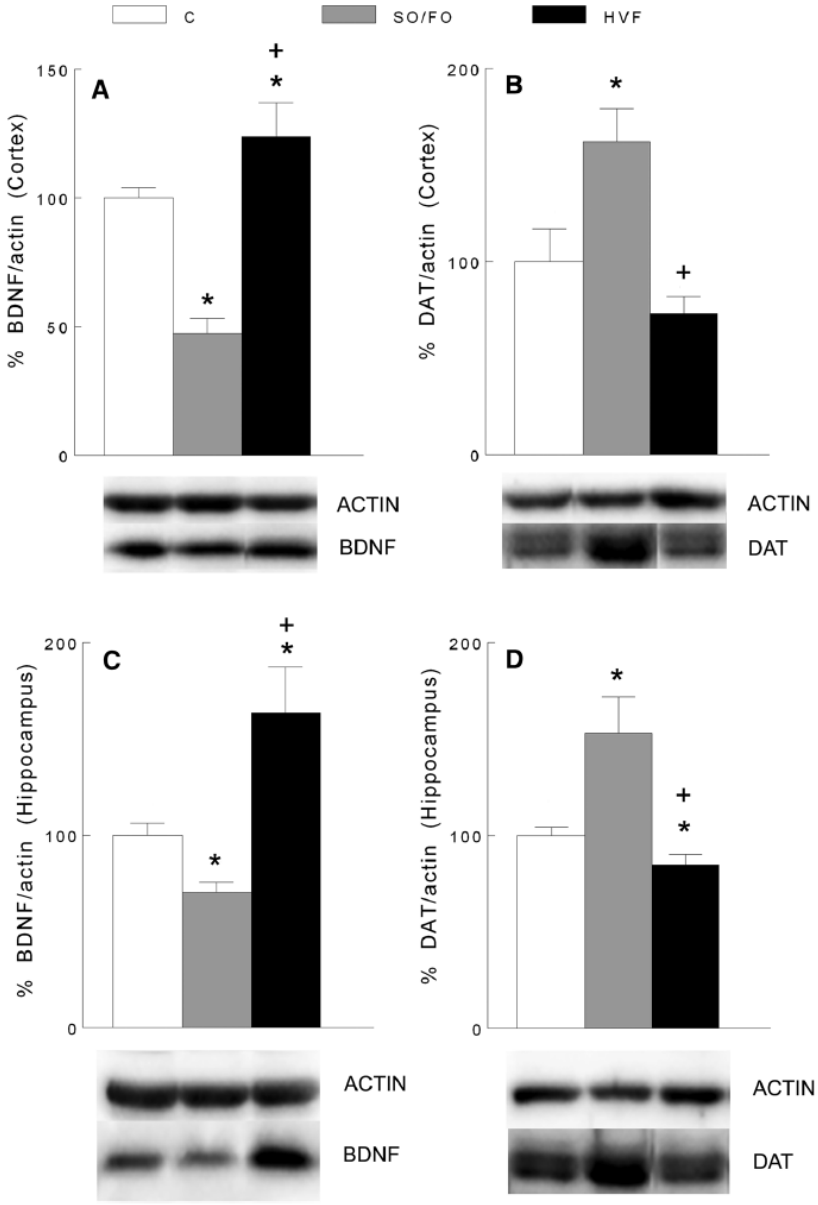

Fig. 5 Level of BDNF in cortex (**a**) and hippocampus (**c**) and level of DAT in cortex (**b**) and hippocampus (**d**) in groups supplemented with water, SO/FO and HVF, respectively. Values are expressed as mean ± S.E.M. ($n = 5$). *Asterisk* indicates significant difference from control (C group), *plus* indicates significant difference from SO/FO; (ANOVA followed by Duncan's test; $P < 0.05$). *BDNF* brain-derived neurotrophic factor, *DAT* dopamine transporter, *C* control, *SO* soybean oil, *FO* fish oil, *HVF* hydrogenated vegetable fat, *AMPH* amphetamine

increased TFA incorporation in both the cortex and the hippocampus (0.84 and 0.46 %, respectively).

Discussion

Young rats born of dams supplemented with different fatty acids from the pre-gestational period and maintained in the same supplementation until the 50th post-natal day showed different reinforcement parameters through the AMPH self-administration paradigm. The current study shows that *trans* fat-supplemented animals were more susceptible to AMPH self-infusions, since this experimental group showed a higher number of lever pressings, indicating an increased dose of AMPH in relation to the other experimental groups. In addition, this drug-seeking behavior occurred along with anxiety-like symptoms and locomotor agitation, as observed in EPM and OF, respectively. In fact, recent studies by our group have shown that *trans* fat supplementation crossover generations could modify behavioral and biochemical parameters related to emotionality and stress (Pase et al. 2013), also facilitating preference for psychostimulant drugs (Kuhn et al. 2013, 2015). Besides these parameters, molecular markers in the cortex (Trevizol et al. 2014) and the hippocampus related to an animal model of mania

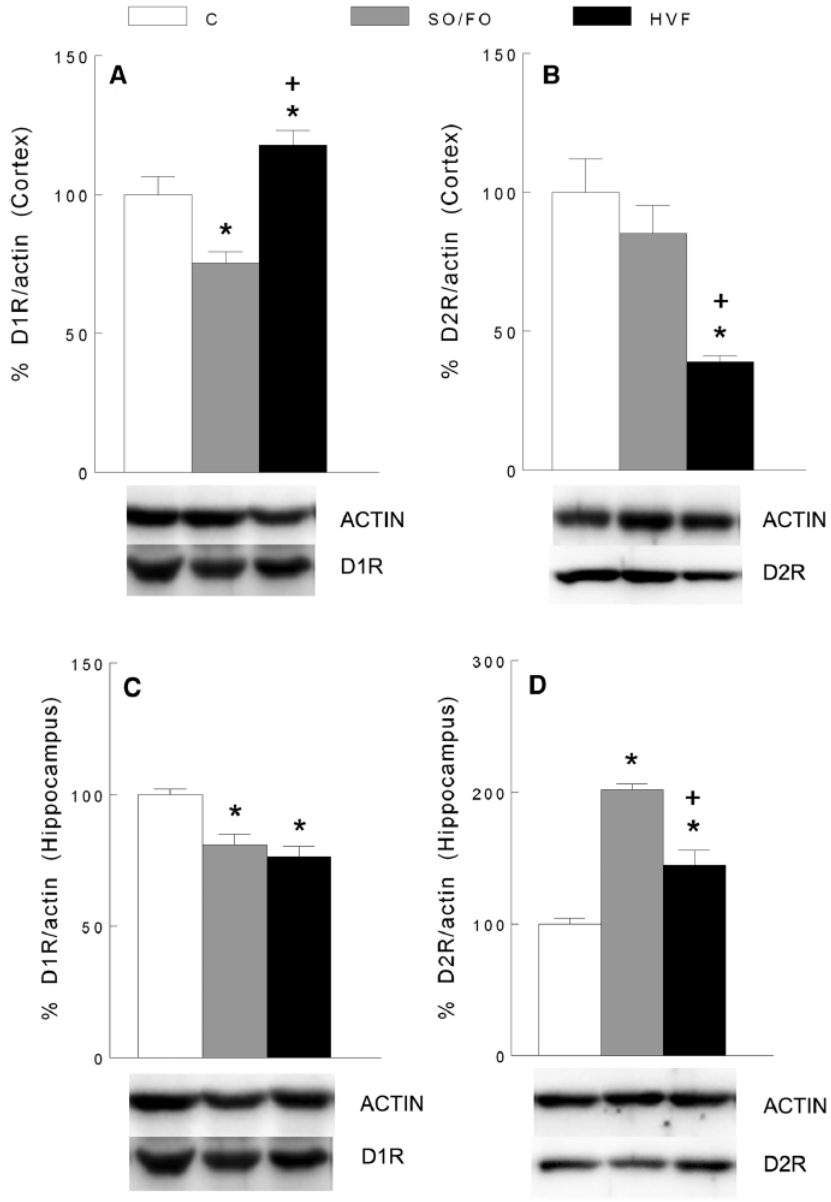

Fig. 6 Level of D1R in cortex (**a**) and hippocampus (**c**) and level of D2R in cortex (**b**) and hippocampus (**c**) in groups supplemented with water, SO/FO and HVF, respectively. Values are expressed as mean ± S.E.M. ($n = 5$). *Asterisk* indicates significant difference from control (C group), *plus* indicates significant difference from SO/FO; (ANOVA followed by Duncan's test; $P < 0.05$). *D1R* dopamine receptor D1, *D2R* dopamine receptor D2, *C* control, *SO* soybean oil, *FO* fish oil, *HVF* hydrogenated vegetable fat, *AMPH* amphetamine

were significantly modified by chronic intake of *trans* fat, indicating that the increased supply of these FA in critical periods of brain development may modify neurotransmission and functionality of brain neurons. In fact, our current findings also showed a significant incorporation of TFA in both the cortex and the hippocampus, suggesting that this change may induce alterations in fluidity and plasticity of neural membranes, as previously suggested (Kuhn et al. 2015, 2013; Trevizol et al. 2014, 2013). Thus, this study has also allowed us to observe that HVF supplementation crossover initial development of life is able to affect brain molecular markers: HVF group showed increased BDNF expression in the hippocampus, being these findings in accordance with the literature, since an up-regulation of BDNF is related to addiction to psychostimulants such as AMPH, cocaine, and nicotine (Lang et al. 2007). Of particular importance, a recent study has shown attenuated rewarding properties to cocaine in partial knockout rats of BDNF gene (Laurent et al. 2013), indicating a relationship between this molecular factor and addiction. Indeed, during the last few years, some studies have focused on the effects of addictive drugs on neurogenesis (Venkatesan et al. 2011; Teuchert-Noodt et al. 2000). Based on the current findings, it is possible to infer that chronic intake of *trans* fat crossover developmental periods of life may facilitate the addiction to psychostimulant drugs, negatively interfering on the neurogenic process, even though preference for

Table 2 Fatty acids composition of cortex and hippocampus of rats supplementing with different oil/fat over first generation (% of total fatty acids identified)

Mean (±SEM)				
Tissue	Fatty acid	C	SO/FO	HVF
Cortex	Σ PUFA	29.67 ± 0.75	30.73 ± 0.56	31.18 ± 0.65
	Σ *n*-3	11.73 ± 0.38[b]	13.84 ± 0.13[a]	12.2 ± 0.32[b]
	Σ *n*-6	17.15 ± 0.41[b]	16.18 ± 0.52[b]	18.31 ± 0.24[a]
	Σ TFA	n.d.	n.d.	0.84 ± 0.03
	*n*6/*n*3 PUFA ratio	1.46 ± 0.00[a]	1.17 ± 0.00[b]	1.5 ± 0.02[a]
Hippocampus	Σ PUFA	29.57 ± 0.38	28.61 ± 1.76	28.84 ± 1.09
	Σ *n*-3	11.08 ± 0.11	11.67 ± 1.79	11.04 ± 1.4
	Σ *n*-6	17.73 ± 0.16	16.29 ± 0.72	17.17 ± 0.33
	Σ TFA	n.d.	n.d.	0.46 ± 0.06
	*n*6/*n*3 PUFA ratio	1.6 ± 0.04[b]	1.47 ± 0.255[b]	1.94 ± 0.04[a]

C control, *SO/FO* soybean oil plus fish oil, *HVF* hydrogenated vegetable fat, *PUFA* polyunsaturated fatty acids, *TFA trans* fatty acids

[a,b] Different lowercases indicate significant difference among C, SO/FO, and HVF in the same brain area ($P < 0.01$)

psychostimulants may also depend on drug dose and administration protocol applied (Gonçalves et al. 2014).

AMPH is a highly neurotoxic and powerful stimulant drug that has steadily gained popularity worldwide, causing irreversible damage to brain cells, especially for its ability to compromise the blood–brain barrier (BBB) function (Bowyer and Ali 2006), leading to neurological, and psychiatric abnormalities that may aggravate the addiction condition. Besides BDNF, HVF supplementation was able to change another important molecular marker, the dopamine transporter (DAT), whose expression decreased in the hippocampus, showing also an interesting tendency to lower in the cortex. Contrarily, SO/FO supplementation was related to an increased DAT expression in both the cortex and the hippocampus, indicating a favorable influence for regulation of dopamine (DA) levels. In this sense, the constitution of phospholipid bilayer of presynaptic nerve endings appears to be able to alter the function of transmembrane transporters, since they are anchored in these terminals (Kodas et al. 2002). When nerve endings are associated with the dopaminergic system, such structural change may affect the regulating function of DA neurotransmission through DAT. Accordingly, the reinforcing properties of AMPH are closely linked to its capacity to increase extracellular dopamine (DA) levels. In fact, AMPH inhibits DA re-uptake, being acknowledged to induce a reversal of this transport, leading to an efflux of DA, which is mediated by dopamine transporter (DAT) (Sulzer 2011; Sitte and Freissmuth 2010). In this sense, competition for uptake and reverse transport leads to an increased extracelullar concentration of DA, which is related to rewarding properties (Schultz 2002). Thus, reverse transport is promoted to facilitate AMPH uptake via DAT, as well as their passive diffusion through the membrane, since it has a lipophilic nature (Sitte et al. 1998; Sandtner et al. 2013). Therefore, when modified, lipids of neural membranes may alter the activity of DAT (Sitte and Freissmuth 2015). In this context, we hypothesized that a supply of *trans* fatty acids (TFA) may impair the fluidity of neural membranes, interfering on the functionality of DAT, which may be less able to control the excess of AMPH-induced DA.

Regarding the molecular markers, our study also showed a significant influence of the supplementations on expression of DA receptors, when HVF supplementation was related to an increased density of D1 receptors in the brain cortex, whereas expression of this receptor was decreased in the hippocampus. Additionally, SO/FO supplementation was related to a down-regulation of D1 receptors in both the cortex and the hippocampus. Inversely, expression of D2 receptors was decreased by *trans* fat in the cortex, and increased by both HVF and SO/FO in the hippocampus, indicating that this subtype of receptor may be inversely affected in different brain areas. Interestingly, literature data have suggested that both D1 and D2 receptors appear to be differently activated during self-administration of psychostimulant drugs, since activation of D1 receptors seems to exert a permissive role on the self-administration activity, whereas D2 receptor mediates reward effects due to reinforcement (Phillips et al. 1994; Maldonado et al. 1993; Beninger et al. 1989). In fact, our findings are not in complete agreement with these hypotheses; however, our results are consistent with the first idea, since the presence of *trans* FA in dopaminergic neuronal membrane phospholipids could contribute to increase the density of D1 receptors in the cortex, exacerbating seeking behavior for psychostimulants. Furthermore, a low ratio of *n*-6/*n*-3 PUFA, as observed in SO/FO group could contribute to a

higher fluidity of neuronal membranes, thereby modulating expression of D1 receptors in this brain area. Considering the influence of different supplementations on dopaminergic receptors in the hippocampus, our findings are less precise: while D1 receptors expression was decreased in both SO/FO and HVF groups, expression of D2 receptors was more strongly increased in SO/FO, and in HVF group in a minor proportion. Literature contributions showed that D2 receptors activation has been related to a decreased DA release, thus affecting locomotor activity (Jackson and Westlind-Danielsson 1994). Our findings partly support these data, since SO/FO group showed higher expression of D2 receptors in the hippocampus, thus disfavoring DA release. Some studies have shown that the two subtypes of DA receptors may adapt to environmental pressure or supplementation in different or even opposite ways, suggesting a possible adaptive role of this functional independence in brain tissues (Puglisi-Allegra et al. 1991; Naef et al. 2008).

As the central nervous system has basic nutritional needs of PUFA, many factors are associated with deficiency of PUFA, especially by increasing the susceptibility for development of affective disorders (Buydens-Branchey et al. 2008). It is known that a decreased intake of PUFA can increase the vulnerability to addictive disorders and stimulating psychostimulant-induced aggressiveness in humans (Buydens-Branchey et al. 2003). PUFA is functionally involved in several neurotransmitter systems, including the dopaminergic system (Zimmer et al. 2000; Chalon 2006, Vines et al. 2012), which is closely related to addiction.

Conclusion

According to the literature, *trans* fats are present in numerous processed foods, whose consumption can modify the plasticity and permeability of brain neural membranes. Thus, the current study contributes to a warning related to public health, since it is showing the harmful influence of *trans* fats on behavioral aspects involved in anxiety-like symptoms and addiction, together with molecular markers such as BDNF and DAT that involve the dopaminergic system, being able to facilitate the addiction potential to psychostimulant drugs such as AMPH.

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Compliance with Ethical Standards

Conflict of interest Authors report no conflict of interest.

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4 DISCUSSÃO

Através dos resultados experimentais obtidos neste estudo que utilizou dois diferentes protocolos experimentais, vemos que:

1. O consumo crônico de AGT ofertado através da suplementação de GVH durante os períodos de desenvolvimento fetal e de crescimento dos animais, durante duas gerações, é capaz de alterar parâmetros de preferência pela anfetamina, sugerindo que tal influência pode modificar a resposta hedônica frente à drogas psicoestimulantes, e facilitando assim o potencial de abuso destas drogas;

2. A suplementação com GVH levou a um aumento nos parâmetros de estresse oxidativo medido através do aumento dos níveis de proteínas carboniladas, e uma diminuição de enzimas antioxidantes, indicando que o consumo de gorduras *trans* provoca uma elevação na geração do processo pró-oxidante verificado;

3. O consumo crônico de GVH aumentou alguns parâmetros de adicção pela anfetamina evidenciado através da autoadministração da droga, mostrando que o AGT pode ter modificado a neurotransmissão dopaminérgica nesse grupo levando a um aumento na autoadministração e elevação dos níveis de BDNF, diminuição da expressão do DAT e uma alteração nos níveis de receptores D1 e D2;

4. Em contraste, os animais suplementados com OP e com a mistura de OS/OP mostraram uma diminuição dos parâmetros de preferência e da frequência de autoadministração da droga, indicando que os AG n-3 podem ter sido capazes de prevenir o aumento dos efeitos de busca pela droga nesses animais bem como manter os níveis de BDNF e elevar a expressão do DAT controlando os níveis de dopamina no córtex e hipocampo;

5. A suplementação de OP mostrou potencial neuroprotetor, observado através dos menores danos oxidativos e também pela maior proteção antioxidante, a qual foi superior em relação ao grupos suplementados com OS e GVH.

6. O consumo crônico de GVH elevou os parâmetros de ansiedade observados após a exposição à ANF, o que sugere uma exacerbação de sintomas de abstinência no período pós-droga nesses animais;

5 CONCLUSÕES FINAIS

Através deste estudo descobrimos que o consumo de gordura *trans* através de gerações, bem como grandes quantidades de ácidos graxos ômega-6 pode favorecer sintomas de dependência pela anfetamina, juntamente com o aumento de sintomas associados a ansiedade e elevação da atividade locomotora. De acordo com a literatura, as gorduras *trans* estão presentes em numerosos alimentos processados, cujo consumo pode modificar a plasticidade e permeabilidade das membranas neuronais do cérebro.

Assim, o presente estudo contribui em relação a saúde pública gerando um alerta para a sociedade, uma vez que ele está mostrando a influência nociva do consumo gorduras *trans* sobre aspectos comportamentais envolvidos em sintomas de dependência e ansiedade, juntamente com alterações nos marcadores moleculares tais como o BDNF e o TDA que envolvem modificações no sistema dopaminérgico, sendo capaz de facilitar a potencial dependência de drogas psicoestimulantes.

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