UNIVERSIDADE FEDERAL DE PERNAMBUCO – UFPE
CENTRO DE CIÊNCIAS DA SAÚDE – CCS
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROPSIQUIATRIA E CIÊNCIAS DO COMPORTAMENTO – POS NEURO – UFPE

ERALDO FONSECA DOS SANTOS JUNIOR

Impacto da desnutrição provocada por grandes ninhadas durante a lactação sobre o estado oxidativo e inflamatório do cólon

RECIFE
2015

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Dissertação apresentada ao Programa de Pós-Graduação em Neuropsiquiatria e Ciências do Comportamento como requisito básico para obtenção do Título de Mestre em Neurociências da Universidade Federal de Pernambuco.
Orientadora: **Profa Dra Belmira Lara da Silveira Andrade da Costa**
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RECIFE
2015

Ficha catalográfica elaborada pela
Bibliotecária: Mônica Uchôa, CRB4-1010

S237i Santos Junior, Eraldo Fonseca dos.
Impacto da desnutrição provocada por grandes ninhadas durante a lactação sobre o estado oxidativo e inflamatório do cólon / Eraldo Fonseca dos Santos Junior. – Recife: O autor, 2015.
149 f.: il.; 30 cm.

Orientadora: Belmira Lara da Silveira Andrade da Costa.
Dissertação (mestrado) – Universidade Federal de Pernambuco, CCS. Programa de Pós-Graduação em Neuropsiquiatria e Ciências do Comportamento, 2015.
Inclui referências, apêndices e anexos.

1. Cólon. 2. Desnutrição. 3. Estresse oxidativo. 4. Glia mioentérica. 5. Sistema nervoso. I. Costa, Belmira Lara da Silveira Andrade da (Orientadora). II. Título.

612.665 CDD (22.ed.) UFPE (CCS2015-089)

ERALDO FONSECA DOS SANTOS JUNIOR

IMPACTO DA DESNUTRIÇÃO PROVOCADA POR GRANDES NINHADAS DURANTE A LACTAÇÃO SOBRE O ESTADO OXIDATIVO E INFLAMATÓRIO DO CÓLON.

Dissertação apresentada ao Programa de Pós-Graduação em Neuropsiquiatria e Ciências do Comportamento da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Mestre em Neurociências.

Dissertação Aprovada em: 26/02/2015

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Aos meus pais Eraldo e Luciana, por
acreditarem e me fazer acreditar que a educação
é o melhor caminho.

AGRADECIMENTOS

Agradeço a Deus, que através da minha família, meus amigos e colegas de laboratório, se fez presente e me ajudou sempre que precisei na realização das minhas atividades. A ele toda honra e glória!

Aos meus pais, por todo amor, dedicação, apoio e por todos os ensinamentos. Por abrir mão de tantas coisas para fazer da educação e da dignidade o maior legado para mim e minha irmã.

À minha orientadora, Profª Belmira, pela sabedoria, competência e honestidade. Por ter me ensinado muito mais do que eu precisasse aprender, sendo uma pessoa de caráter único, que me orientou no sentido amplo da palavra. Obrigado!

Agradecimento especial para minha co-orientadora, Catarina, por me ajudar e contribuir imensamente para o meu aprendizado.

À minha namorada, Lorena, pelo companheirismo e paciência. Por ser a melhor namorada não cientista que um cientista pode ter.

Agradeço aos meus colegas de laboratório que me acompanharam nesses últimos anos: Alinny, Ricielle, Giselle, Raone, Emerson, Lucas, Thiago, Aluizio, Soledade, Ana Paula, Bira, Manu e David. E em especial aqueles que estiveram comigo de maneira mais próxima: Renata Vaz, Renata Santos, Igor e Cida, com certeza tornaram meus dias mais leves, obrigado!

Ao Profº Marcelo Rodrigues e a Profª Angela Santos, que me ajudaram sempre que precisei.

Aos técnicos Nielson Melo e Dijanah Machado, a veterinária Cláudia e ao seu Fredson, pela ajuda diária nessa jornada.

Aos diversos laboratórios e seus integrantes, que viabilizaram a realização dessa pesquisa.

Aqueles animais, solicitadas pela ciência em benefício da humanidade, o meu respeito e eterna gratidão.

A FACEPE, pelo apoio financeiro.

“Nada me perturbe, nada me amedronte.

Tudo passa. Só Deus não muda.

A paciência tudo alcança.”

(Santa Teresa D´Ávila)

RESUMO

O sistema nervoso entérico (SNE) é a inervação intrínseca do trato gastrointestinal, envolvida no controle autonômico das suas atividades motoras e secretórias. Evidências recentes indicam que a glia do SNE participa ativamente na manutenção da homeostase intestinal, mas pouco se sabe sobre a sua função em quadros de desnutrição. Neste trabalho, avaliamos se a desnutrição provocada por reduzida ingestão de leite materno é capaz de comprometer a organização estrutural do SNE, balanço redox, reserva antioxidante e a condição anti-inflamatória do cólon, principalmente associadas à função da glia. Ratos wistar foram criados em diferentes tamanhos de ninhada, com 6 (N6, ninhada normal) ou 15 filhotes (N15, grande ninhada), constituindo o grupo nutrido e o desnutrido, respectivamente, que foram analisados no dia do desmame (25º dia pós-natal). Os animais machos foram pesados semanalmente até o dia do experimento. Sob anestesia profunda, foi obtido o comprimento nasoanal e retirado o cólon distal para análise. Uma parte do material foi utilizada para obtenção de homogenados, os quais foram processados para análise dos níveis de lipoperoxidação, TNFα, IL-1β, IL-10, produção de óxido nítrico (NO), GSH e GSSG e atividade das enzimas superóxido dismutase total (tSOD) e catalase (CAT). Outra parte do material foi utilizada para análises morfométricas do cólon e para obtenção de preparações longitudinais do plexo mioentérico, onde a distribuição de neurônios e glia foram avaliadas por imunohistoquímica. Os resultados, expressos em média ± desvio padrão, mostraram diminuição de peso nos animais desnutridos (N6; 50,70 ± 9,77 e N15; 30,13 ± 2,87g), bem como no comprimento nasoanal (N6; 13,41 ± 0,44 e NN15; 11,00 ± 0,42 cm). A espessura do cólon (N6; 1,27 ± 0,09 e N15; 1,09 ± 0,08 cm), da parede total (N6; 1010,05 ± 114,70 e NN15; 905,75 ± 169,77 µm) e da *tunica muscularis* (N6; 84,00 ± 22,30 e N15; 74,02 ± 22,38 µm) também foram afetadas. Do ponto de vista qualitativo, o plexo mioentérico do grupo desnutrido não apresentou alterações na sua estrutura, no entanto, um menor tamanho no corpo celular dos neurônios, foi detectado (N6; mediana 310,85; min 77,96; max 987,37 e NN15 mediana 291,74; min 114,68; max 993,41 μm^2). Maiores níveis de lipoperoxidação (N6; 1,079 ± 0,10 e NN15; 2,51 ± 0,22 nmol MDA/mg proteína) óxido nítrico (N6; 3,04 ± 0,63 e N15; 4,85 ± 1,8 µM/mg proteína) e da atividade da CAT (N6; 0,027 ± 0,010 e N15; 0,049 ± 0,024 U/mg de proteína) foram detectadas no grupo desnutrido, assim como os níveis de TNFα (N6 1,26 ± 0,26 e N15; 1,57 ± 0,24 pg/mg de proteína) e IL-1β (N6 0,88 ± 0,15 e N15; 1,17 ± 0,13 pg/mg de proteína). Em relação aos níveis de IL-10 (N6 44,54 ± 13,09 e N15; 51,22 ± 8,15 pg/mg de proteína) e GSH (N6 60,89 ± 21,26 e N15; 53,34 ± 26,19 µM/mg de proteína) não observamos diferença entre os grupos, embora os níveis de tSOD (N6 0,088 ± 0,023 e N15; 0,056 ± 0,018 U/mg de proteína) e GSSG se apresentaram diminuídos no grupo experimental (N6 27,48 ± 4,59 e N15; 14,41 ± 4,12 µM/mg de proteína). Os resultados sugerem que reduzido aporte de nutrientes durante a lactação reduz alguns dos mecanismos de proteção relacionados com a reserva anti-oxidante e anti-inflamatória, o que poderá aumentar a vulnerabilidade do cólon a insultos externos.

Palavras–chave: Cólon. Desnutrição. Estresse oxidativo. Glia mioentérica. Sistema nervoso entérico.

ABSTRACT

The enteric nervous system (ENS) is the intrinsic innervation of the gastrointestinal tract involved in the autonomic control of motor and secretory activity. Recent evidences indicates that glia from ENS actively participates in maintaining intestinal homeostasis, but little is known about its function in malnutrition situations. In this work, we evaluate the malnutrition caused by reduced intake of breast milk can compromise the structural organization of the ENS, redox balance, antioxidant reserve and the anti-inflammatory condition of the colon, mainly associated with the role of glia. Wistar rats were raised in different litter sizes, with 6 (N6, regular litter) or 15 puppies (N15, large litter), constituting the large group and the malnourished, respectively, were analyzed on the day of weaning (25th postnatal day). The male animals were weighed weekly until the day of the experiment. Under deep anesthesia was obtained nasoanal length and the distal colon removed for analysis. Part of the material was used to obtain a homogenate, which were processed for analysis of the levels of lipid peroxidation, TNFα, IL-1β, IL-10, nitric oxide (NO), GSH and GSSG and enzyme activity superoxide dismutase total (tSOD) and catalase (CAT). Another part of the material was used for morphometric analysis of colon and to obtain longitudinal preparations of the myenteric plexus where the distribution of neurons and glia were evaluated by immunohistochemistry. The results, expressed as mean ± standard deviation, showed decreased weight in malnourished animals (N6; 50.70 ± 9.77 and N15; 30.13 ± 2.87g) and the nasoanal length (N6; 13.41 and NN15 ± 0.44, 11.00 ± 0.42 cm). The thickness of the colon (N6, N15, and 1.27 ± 0.09, 1.09 ± 0.08 cm), the total wall (N6; 1010.05 ± 114.70 and NN15; 905.75 ± 169.77 microns) and the *tunica muscularis* (N6; 84.00 ± 22.30 and N15; 74.02 ± 22.38 microns) were also affected. From a qualitative point of view, the myenteric plexus of the malnourished group had no change in its structure, however, a smaller size in the cell body of neurons was detected (N6; median 310.85; 77.96 min; max 987.37 and NN15 median 291.74; 114.68 min, max 993.41 μm2). Higher levels of lipid peroxidation (N6; 1.079 ± 0.10 and NN15, 2.51 ± 0.22 nmol MDA / mg protein) nitric oxide (N6, 3.04 ± 0.63 and N15; 4.85 ± 1.8 uM / mg protein) and CAT activity (N6, N15, and 0.027 ± 0.010, 0.049 ± 0.024 U / mg protein) was detected in malnourished group, and TNFα levels (1.26 ± 0.26 and N6 N15; 1.57 ± 0.24 pg / mg protein) and IL-1β (N6 and N15 0.88 ± 0.15, 1.17 ± 0.13 pg / mg protein). Regarding IL-10 levels (44.54 ± 13.09 N6 and N15; 51.22 ± 8.15 pg / mg protein) and GSH (N6 and N15 60.89 ± 21.26, 53.34 ± 26.19 uM / mg protein) we found no difference between the groups, although the levels of tSOD (0.088 ± 0.023 N6 and N15; 0.056 ± 0.018 U / mg protein) and GSSG performed decreased in the experimental group (27 N6 48 and N15 ± 4.59; 14.41 ± 4.12 uM / mg protein). The results suggest that reduced supply of nutrients during lactation reduces some of the protection mechanisms related to the anti-oxidant and anti-inflammatory reserve, which may increase the vulnerability of the colon to external insults.

Keywords: Colon. Enteric nervous system. Malnutrition. Myenteric glia. Oxidative stress.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

- ✓ **CAT-** Catalase
- ✓ **CGE –** Células Gliais Entéricas
- ✓ **CN –** Crista neural
- ✓ **GFAP** - Proteína Glial Fibrilar Ácida
- ✓ **GSH -** Glutationa Reduzida
- ✓ **GSSG -** Glutationa Oxidada
- ✓ **HE –** Hematoxilina Eosina
- ✓ **HuC/HuD –** Marcador pan-neuronal entérico
- ✓ **N15 –** Ninhada com 15 filhotes, grupo desnutrido
- ✓ **N6 –** Ninhada com 6 filhotes, grupo nutrido
- ✓ **NO –** Óxido nítrico
- ✓ **PBS –** Tampão fosfato salina
- ✓ **SNC –** Sistema Nervoso Central
- ✓ **SNE –** Sistema nervos Entérico
- ✓ **TBARs –** Substâncias reativas ao ácido tiobarbitúrico
- ✓ **TFO –** Tampão fosfato
- ✓ **TGI –** Trato gastrointestinal
- ✓ **tSOD –** Superóxido dismutase total

SUMÁRIO

1. INTRODUÇÃO

1.1 Trato gastrointestinal – Cólon

O trato gastrointestinal (TGI) é basicamente um tubo muscular com epitélio especializado que compreende a cavidade bucal, faringe, esôfago, estômago, intestino delgado e grosso, e ânus. Além disso, existem os órgãos anexos, tais como: glândulas salivares, fígado e pâncreas, os quais secretam substâncias que auxiliam no processo digestivo dos alimentos. O TGI, juntamente com os órgãos anexos, tem a função de receber, digerir, absorver e eliminar substâncias ingeridas (GUYTON; HALL, 2002; MERCHANT, 2007).

Nos mamíferos, o intestino grosso contribui para o balanço hidroeletrolítico e absorção de nutrientes, controla a velocidade de formação e eliminação das fezes e é o habitat de milhões de microorganismos (GUYTON; HALL, 2002). O cólon é caracterizado por ter uma mucosa com epitélio de revestimento do tipo colunar prismático com uma fina borda estriada (microvilos). A lâmina própria é rica em linfócitos e nódulos linfáticos, que frequentemente atravessam a muscular da mucosa, invadindo a submucosa. Na submucosa são encontrados neurônios e células da glia pertencentes ao plexo submucoso do sistema nervoso entérico (SNE). A camada muscular é bem desenvolvida e possui duas subcamadas de fibras, uma circular e outra longitudinal, entre as quais estão localizados os plexos mioentéricos do SNE. A serosa é constituída por tecido conjuntivo frouxo(CARNEIRO; JUNQUEIRA, 2013).

O intestino grosso do rato é composto pelo ceco, cólon proximal (ascendente), cólon distal (descendente) e reto. Diferenças funcionais, relacionadas principalmente com o peristaltismo, são descritas entre as duas porções do cólon. No cólon proximal é encontrado um antiperistaltismo que possibilita reter o material fecal por um tempo maior, aumentando a capacidade de mistura, armazenagem e a absorção do excesso de fluidos. Já nó cólon distal é observada uma intensa contração peristáltica que resulta na propulsão das fezes já desidratadas (GUYTON; HALL, 2002; HASLER; KUROSAWA; CHUNG, 1990; MESSENGER; BORNSTEIN; FURNESS, 1994)

1.2 Sistema nervoso entérico – O segundo cérebro

O sistema nervoso entérico (SNE), atualmente, é reconhecido como uma divisão própria do sistema nervoso autônomo, juntamente com os sistemas nervoso simpático e parassimpático, chegando a possuir cerca de 600 milhões de neurônios em humanos (FURNESS, 2000, 2012) e uma variedade de neurotransmissores e neuromoduladores semelhantes aos encontrados no sistema nervoso central (FURNESS, 2012). O SNE se estende desde o esôfago até o esfíncter anal externo, incluindo, ainda, elementos nervosos da vesícula biliar e ducto pancreático (FURNESS, 2000).

Ele tem a capacidade de mediar atividades de forma independente do SNC, e inclui uma série de redes neurais, necessárias para um circuito reflexo completo (CABARROCAS; SAVIDGE; LIBLAU, 2003; FURNESS, 2006, 2012; LARANJEIRA; PACHNIS, 2009). O mesmo é constituído por um vasto número de neurônios com diversas morfologias e fenótipos neuroquímicos, e células gliais, que estão organizados em dois plexos ganglionares, o plexo mioentérico e o plexo submucoso (Figura 1) (FURNESS, 2006, 2012; PHILLIPS et al., 2004).

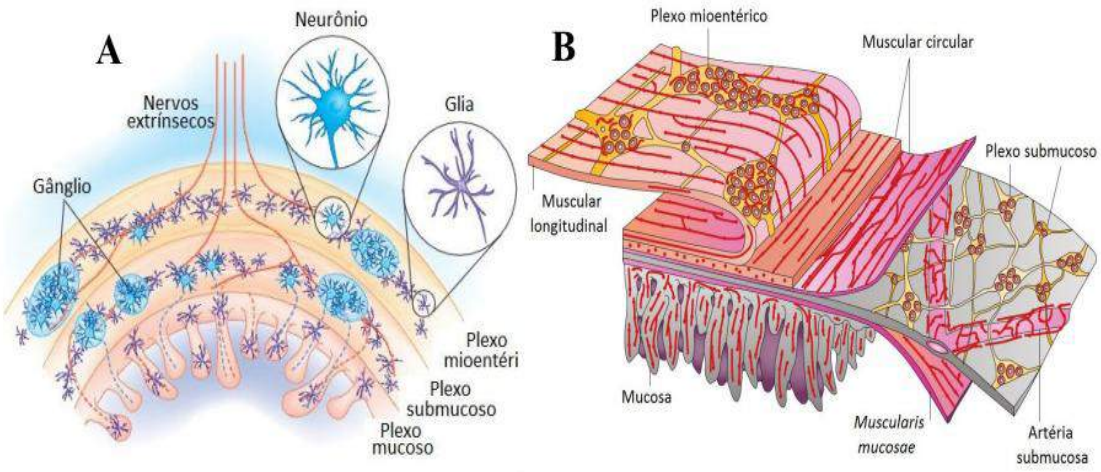

Figura 1. Organização do sistema nervoso entérico em secção transversal (A) e em camadas (B). Figura adaptada de Nature publishing group, 2008 (A) e Furnnes, 2012 (B).

O plexo mioentérico (plexo de Auerbach), que regula essencialmente a função motora, localiza-se entre a camada muscular longitudinal externa e a camada do músculo circular, presente por todo trato digestório, do esôfago ao reto (FURNESS, 2006). O plexo submucoso (de Meissner) é proeminente no intestino delgado e grosso e divide-se em plexo submucoso interno, abaixo da mucosa, plexo submucoso externo, junto à camada circular do músculo e o

plexo intermediário posicionado entre os plexos submucoso e externo. Suas malhas são menores que o plexo mioentérico, suas fibras interconectadas são mais finas e o gânglio é menor. Este plexo localiza-se ao longo do intestino, sendo que um plexo fica próximo do músculo e o outro próximo da mucosa (FURNESS, 2006, 2012).

A rede neural do plexo mioentérico está envolvida na regulação reflexa das atividades contráteis da musculatura externa, enquanto que a rede neural do plexo submucoso tem um papel direto no controle de secreção e absorção, através dos neurônios motores que regulam a atividade secretomotora e vasomotora da mucosa (LOMAX; FURNESS, 2000; SONG; BROOKES; COSTA, 1994). Os plexos entéricos seguem um padrão ao longo do trato digestório, porém diferenças quanto à densidade e ao tamanho dos neurônios, bem como a forma dos gânglios, podem ser encontrados no mesmo segmento do trato gastrintestinal dos animais de mesma espécie e com diferentes idades (MATINI; MAYER; FAUSSONE-PELLEGRINI, 1997; MCKEOWN; CHOW; YOUNG, 2001)

1.3 Células gliais entéricas

Células gliais entéricas (CGE) constituem uma grande população de células da glia periférica, superam os neurônios entéricos numa proporção de 4:1 e estão localizadas dentro dos gânglios do plexo mioentérico e submucoso do SNE, e em locais extraganglionares, como camada muscular lisa e mucosa intestinal (GERSHON; ROTHMAN, 1991; GULBRANSEN; SHARKEY, 2012; RUHL, 2005).

A aparência estrelada e a associação física íntima entre glia e os neurônios entéricos é bastante parecida com a relação entre astrócitos e neurônios do sistema nervoso central (GULBRANSEN et al., 2012). Com efeito, a glia entérica e os astrócitos são também similares ao nível molecular, dado que compartilham propriedades eletrofisiológicas (HANANI et al., 2000, OLSSON, 2010) e expressam proteínas semelhantes, incluindo o filamento intermediário glial da proteína fibrilar ácida (GFAP) e vimentina (RÜHL; NASSER; SHARKEY, 2004). Além do GFAP, as CGE também expressam a proteína S100, que é uma proteína ligante de cálcio, considerada um marcador pan-glial no SNE, e tem algumas funções, como a regulação da estrutura e função do citoesqueleto e a homeostase de cálcio no citoplasma das CGE (FERRI et al., 1982; RÜHL, 2005b)

Uma série de estudos tem demonstrado que CGE são fundamentais para a regulação da motilidade intestinal, funções secretoras e de absorção do epitélio intestinal e de defesa do

hospedeiro contra patógenos (BASSOTTI et al., 2007; DE GIORGIO et al., 2012; GULBRANSEN; SHARKEY, 2012; SORET et al., 2013). As CGE possuem a capacidade de criar um microambiente protetor, por meio de tamponamento do meio extracelular, via absorção de cátions (RÜHL, 2005; RÜHL; NASSER; SHARKEY, 2004) e liberação de fatores tróficos como, fator neurotrófico derivado de célula glial (GDNF), que está relacionada com o desenvolvimento e sobrevivência de neurônios entéricos (BASSOTTI et al., 2007; RODRIGUES et al., 2011). Estão relacionadas com a neurotransmissão, devido à expressão de glutamina sintetase, que tem um papel na sinalização glutamatérgica e L-arginina (um importante precursor de óxido nítrico), que tem um papel na sinalização nitrérgica e expressão de purinoreceptores como P2Y4 e P2X7 (BASSOTTI et al., 2007; VANDERWINDEN et al., 2002).

A resposta do sistema nervoso para diversas lesões acarreta na ativação das células gliais entéricas (LOPACHIN; ASCHNER, 1993; O'CALLAGHAN, 1993). Essa ativação tem sido sugerida como um mecanismo de sinalização precoce, responsável pela degeneração neuronal subsequente (GIULIAN; LI; KEENEN, 1994). Estudos têm demonstrado que as anormalidades estruturais ou bioquímicas das próprias células gliais entéricas poderiam contribuir nas desordens gastrintestinais, e que isto poderia atrair células imunes para o SNE e com isso, levar a uma neurodegeneração (CABARROCAS; SAVIDGE; LIBLAU, 2003)

1.4 Importância da glia do sistema nervoso entérico para o estresse oxidativo e inflamação

Em 1998, Bush et al. (BUSH et al., 1998), depletaram camundongos adultos de células GFAP+ e observaram que, em apenas duas semanas, todos os animais morreram devido a um quadro de jejunoileíte fulminante. Esse quadro foi independente de processos infecciosos, sendo caracterizado por degeneração de neurônios mioentéricos e hemorragia intestinal. Outra função atribuida a estas células é a de manutenção da integridade da barreira epitelial, entre o lúmen do intestino e as células e tecidos do interior da parede intestinal (SAVIDGE et al., 2007; TOUMI et al., 2003). Recentemente, evidenciou-se que elas também possuem receptores para vários neurotransmissores sendo ativados, por exemplo, pela liberação de ATP a partir de neurônios intrínsecos e extrínsecos após a estimulação química ou elétrica (BOESMANS et al., 2013; GULBRANSEN; BAINS; SHARKEY, 2010; GULBRANSEN; SHARKEY, 2012; VELLOSO et al., 2009). Além de responder a estímulos do ATP, as CGE também respondem as citocinas, possuindo receptores para IL-1, IL-6 e quimiocinas,

como a proteína quimiotática de monócitos 1 (MURAKAMI; OHTA; ITO, 2009; RÜHL; TROTTER; STREMMEL, 2001; STOFFELS et al., 2014). Foi demonstrado que em cultura, sob influência de citocinas pró-inflamatórias, células gliais GFAP- podem se tornar GFAP+ (VON BOYEN et al., 2004).

Os mecanismos pelos quais a glia entérica protege a integridade intestinal estão começando a aparecer e várias linhas sugerem que seja de natureza multifatorial. Tem sido sugerido que o fator neurotrófico derivado das células gliais (GDNF) elevado durante a inflamação pode agir para proteger as células da apoptose, (BASSOTTI et al., 2006; STEINKAMP et al., 2012) assim como a síntese de glutationa reduzida por parte da glia pode minimizar os efeitos do estresse oxidativo sobre o dano neuronal (ABDO et al., 2010). Em condições normais, a integridade da mucosa intestinal fica sobre um delicado controle dado pela produção de citocinas pró- e anti-inflamatórias (BOSANI; ARDIZZONE; PORRO, 2009). A reação inflamatória descontrolada na doença de Crohn, por exemplo, provavelmente é o resultado da interação entre deficiência genética e sistema imune inato e uma exagerada resposta da imunidade adaptativa, onde certamente se observa um predomínio da produção de citocinas pró-inflamatórias em detrimento da produção de citocinas anti-inflamatórias (BOSANI; ARDIZZONE; PORRO, 2009). Vários trabalhos associam a causa da doença de Crohn com a perda da função glial (BUSH et al., 1998; CORNET et al., 2001; NEUNLIST et al., 2008; VON BOYEN et al., 2011). A destruição da célula glial entérica por mecanismos autoimunes induz inflamação no intestino (CORNET et al., 2001). Adicionalmente, esses autores descreveram pela primeira vez a diminuição da rede glial entérica em pacientes que sofriam de Doença de Crohn, achado que foi confirmado em um estudo mais recente (VON BOYEN et al., 2011).

A perda da estrutura glial pode ter causas não imunes, como um vírus, ou ainda, uma causa imune, como altos níveis do Fator de Necrose Tumoral (TNF-α) (REINSHAGEN et al., 2000). O TNF-α desempenha um papel importante na patogênese de doenças inflamatórias intestinais, e a terapia anti-TNF-α utilizando anticorpos monoclonais é utilizada como principal alternativa terapêutica (D'HAENS et al., 2011). Por outro lado, a citocina IL-10 suprime a inflamação por vários mecanismos imunológicos, incluindo a redução da expressão de HLA classe II, a redução da secreção de IL-2 pelas células T, e a diminuição de outras citocinas como TNF-α e IL-8. A deficiência do gene da IL-10, em camundongos propicia o desenvolvimento espontâneo de inflamação transmural do intestino. Este tipo de inflamação é

agravado pela presença de bactérias na luz do intestinal e pode ser prevenida com a administração de IL-10 (HERFARTH; SCHÖLMERICH, 2002).

1.5 Importância da presença do alimento durante o desenvolvimento do SNE e maturação em função do alimento

O SNE origina-se a partir da coordenada migração, expansão e diferenciação de células progenitoras da crista neural (CN) vagal e sacral que colonizam uniformemente todo o TGI (HEANUE; PACHNIS, 2007; LAKE; HEUCKEROTH, 2013; SASSELLI; PACHNIS; BURNS, 2012). Durante o desenvolvimento, células da CN vagal entram no esôfago e migram em direção rostro-caudal (Figura 2); colonizando todo o TGI gerando a maior parte do SNE. A CN sacral contribui para um subconjunto de gânglios entéricos no intestino grosso, colonizando o cólon no sentido póstero-anterior. Durante este processo, as células entéricas da CN se auto-renovam e começam a expressar marcadores de linhagens neuronais e gliais conforme vão povoando o intestino (BURNS, 2005).

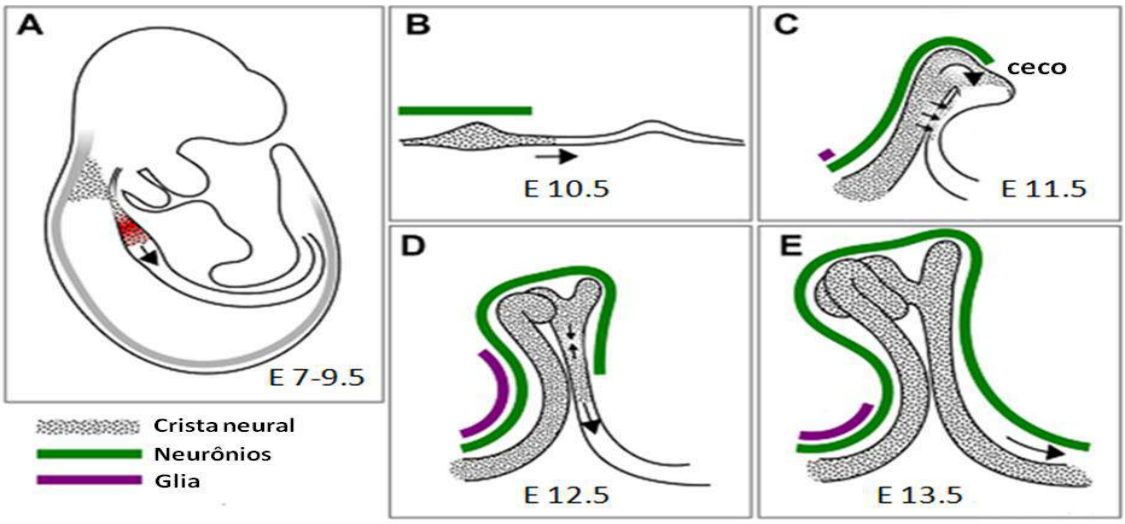

Figura 2. Colonização do trato gastrointestinal do rato pelas células derivadas da crista neural, neurônios e glia, durante o desenvolvimento embrionário (dias embrionários - E). Figura adaptada de Lake & Heuckeroth, 2013.

No embrião, a diferenciação neuronal ocorre antes da diferenciação glial, e nos mamíferos em geral, a diferenciação do fenótipo adulto das células nervosas, bem como a formação dos circuitos neuronais funcionais, ocorre após o nascimento (GOLDSTEIN; HOFSTRA; BURNS, 2013), mostrando assim a importância de uma nutrição adequada no

período pós-natal. Alterações fenotípicas e funcionais ocorridas durante a vida impactam diretamente nas funções gastrointestinais. Embora algumas dessas mudanças sejam geneticamente programadas, as mudanças no meio da parede do intestino e lúmen influenciam diretamente a neuroquímica e a neurobiologia desse sistema. Entre esses fatores estão os nutrientes presentes na dieta (NEUNLIST; SCHEMANN, 2014).

Sendo cada vez mais reconhecido que os nutrientes podem impactar o SNE, alguns poucos trabalhos vem associando situações de desnutrição com a funcionalidade desse sistema. Aumento na densidade neuronal do plexo mioentérico foram observadas (MEILLUS; NATALI; DE MIRANDA NETO, 1998; NATALI; MIRANDA-NETO; ORSI, 2003). Alguns distúrbios e desordens intestinais como Retocolite Ulcerativa Inespecífica e Doença de Crohn, que acometem o TGI, são comumente associadas à desnutrição energético - protéica (BURGOS et al., 2008). O suporte nutricional oral, enteral e parenteral tem se mostrado bastante eficaz na remissão de doenças inflamatórias intestinais, pelo fornecimento de nutrientes com ações fisiológicas específicas. Estes nutrientes atuam modulando a resposta imunoinflamatória e mantendo a integridade da mucosa intestinal, melhorando o estado clínico e, consequentemente, o estado nutricional de pacientes (BURGOS et al., 2008).

Em um trabalho recente, foi visto que a restrição dietética (50% da dieta controle) por cinco meses, em ratos idosos, foi capaz de interferir no estado oxidativo do plasma e na inervação intestinal intrínseca, causando ainda hipertrofia da glia e atrofia do corpo neuronal, além de diminuição na espessura da parede intestinal (CIRILO et al., 2013). Moreira et al., (2012) demonstrou também que uma desnutrição proteica (4% de proteína contra 23% do controle) durante 90 dias, em ratos adultos, foi capaz de atrofiar neurônios mioentéricos do intestino delgado, evidenciados tanto pelo Giemsa (população total) quanto pela NADH-diaforase (subpopulação nitrérgica) (MOREIRA et al., 2012). Outro estudo mostra diminuição do soma e da densidade neuronal, além de uma desorganização na estrutura do plexo do IG de ratos de 21 dias de vida, cujas mães foram submetidas a uma dieta com 0% de caseína desde a gestação (CASTELUCCI et al., 2002).

O estado de alimentação influencia reflexos musculares, por exemplo, a atividade reflexa foi aumentada em segmentos do íleo de animais que foram realimentados após um período de jejum durante a noite, efeito provavelmente impulsionado pelo plexo mioentérico (ROOSEN et al., 2012). Neurônios entéricos expressam transportadores e receptores que estão envolvidos na detecção de nutrientes, permitindo-lhes perceber diretamente e responder

aos mesmos (FURNESS et al., 2013). Além disso, estudos recentes sugerem que os nutrientes também afetam a sobrevivência e proliferação neuronal. Durante a infância, os fatores neurotróficos no leite favorecem a sobrevivência neuronal e crescimento de neuritos (FICHTER et al., 2011). No entanto, a dieta também pode afetar negativamente a sobrevivência de neurônios entéricos, camundongos e ratos adultos alimentados com dietas ricas em gordura perderam neurônios mioentéricos do íleo e do cólon, que foi associado com o trânsito intestinal retardado (VOSS et al., 2013).

Ainda no que tange a alimentação, trabalhos mostram que o leite materno reduz o risco de adquirir diferentes infecções na infância, incluindo os do trato gastrointestinal e respiratório (DEWEY; HEINIG; NOMMSEN-RIVERS, 1995; VILLALPANDO; HAMOSH, 1998). O leite materno contém também um grande número de agentes que conferem proteção contra a infecção, incluindo antimicrobiano, anti-inflamatório, e agentes imunomoduladores que teoricamente poderiam servir como potenciais modificadores ambientais no desenvolvimento de doenças crônicas (BARCLAY et al., 2009; RODRIGUEZ-PALMERO et al., 1999). Existe também evidência direta do efeito de leite materno atuando como um modificador de doença em modelos animais de colites. Em um modelo animal de doença inflamatória intestinal, em camundongos deficientes de IL-10, o leite materno causou uma diminuição na inflamação do cólon em comparação com os controles (MADSEN et al., 2002). Acredita-se, portanto, que a amamentação tem um efeito específico sobre o desenvolvimento inicial de doenças inflamatórias intestinais.

1.6 Modelo animal de desnutrição por grandes ninhadas

Uma nutrição adequada durante a fase inicial de vida dos mamíferos é um fator essencial para assegurar o desenvolvimento normal do indivíduo e estudos epidemiológicos e com animais têm sugerido cada vez mais que uma vida saudável pode ser influenciada por eventos no início da vida (ROCHA-DE-MELO et al., 2004; VELKOSKA et al., 2008). O estado nutricional durante períodos críticos de desenvolvimento influenciam o início da maioria das doenças na vida adulta (MCMILLEN; ADAM; MÜHLHÄUSLER, 2005). A importância da nutrição no início da vida foi inicialmente demonstrada manipulando tamanho das ninhadas em ratos, de tal forma que os ratos de grande ninhadas, receberam menos leite durante a sucção ao longo das 3 primeiras semanas de vida. (MCCANCE, 1962)

O modelo experimental das grandes ninhadas, que induz desnutrição durante o período de aleitamento, vem sendo usado com sucesso (CHAHOUD; PAUMGARTTEN, 2009; DOS SANTOS et al., 2006; VIANA et al., 2013). Existe uma relação inversa entre o peso corporal das crias e o tamanho da ninhada, durante todo o período de aleitamento (CHAHOUD; PAUMGARTTEN, 2009). Alguns parâmetros de maturação como desenvolvimento de peles, aparecimento dos dentes incisivos e abertura dos olhos também estão atrasados em filhotes com o tamanho da ninhada aumentado(CHAHOUD; PAUMGARTTEN, 2009).

Tem sido demonstrado que o tamanho de ninhada afeta os níveis de oxitocina da mãe, bem como a sua produção de leite e os níveis de ejeção (RUSSELL, 1980). Embora a produção de volume de leite aumenta com o tamanho da ninhada , acredita-se que há limites para esse aumento de modo que, acima de um determinado tamanho o fornecimento de leite por filhotes diminuiria. Nessa linha, foi relatado que a produção de leite de ratos não conseguiu mostrar novos aumentos quando o tamanho da ninhada atingia mais de 11 filhotes (KUMARESAN; ANDERSON; TURNER, 1966). Uma vez que há esse limite para a quantidade de leite produzida por mãe, esse método se tornou eficaz em produzir animais desnutridos pré-desmame (HEGGENESS et al., 1961; NAGY; PORADA; ANDERSON, 1977).

Tendo em vista apenas o aleitamento, descartamos qualquer interferência da condição nutricional gestacional das mães. Este modelo experimental tem sido considerado como uma característica que varia, naturalmente, do meio ambiente de desenvolvimento precoce e pode induzir modificações permanentes no cérebro do sistema imune inato de ratos adultos (BIESWAL et al., 2006; VIANA et al., 2013)

2. JUSTIFICATIVA E HIPÓTESE DO PRESENTE ESTUDO

Estudos epidemiológicos têm correlacionado desnutrição com a função gastrointestinal enfraquecida, resposta imune inata e adaptativa depreciadas, prejuízo na barreira intestinal (MAIER et al., 2013; UENO et al., 2011), o aumento na probabilidade de diarréia (DE QUEIROZ et al., 2014) e, recentemente, com defeitos na maturação microbiota intestinal infantil (GORDON et al., 2012). Alguns estudos, inclusive, indicaram a falta de aleitamento materno como fator de risco associado com o desenvolvimento posterior da doença de Crohn no cólon (BARCLAY et al., 2009; DE OLIVEIRA ASSIS et al., 2011; KLEMENT; REIF, 2005; KOLETZKO et al., 1989).

No entanto, a repercussão da baixa quantidade de alimentação durante a lactação no cólon e, em especial sobre as células gliais entéricas, não foi ainda totalmente compreendido. Tendo em mente a importância do período de amamentação para um desenvolvimento adequado do SNE, testamos a hipótese de que o aleitamento desfavorável é capaz de prejudicar a estrutura e função dos neurônios entéricos e células gliais, induzir estresse oxidativo e alterar o estado inflamatório no cólon de ratos lactantes.

3. OBJETIVOS

3.1 Geral

Avaliar em ratos jovens recém-desmamados, os efeitos de reduzido aporte alimentar durante o aleitamento, sobre parâmetros bioquímicos e morfológicos do cólon, relacionados ao desenvolvimento e atividade do sistema nervoso entérico.

3.2 Específicos

1) Acompanhar a evolução ponderal dos animais durante todo o período de experimentação, bem como seu comprimento nasoanal ao fim do período de lactação;

2) Analisar modificações estruturais no cólon distal e plexo mioentérico;

3) Analisar a distribuição e parâmetros morfométricos da glia e neurônios do plexo mioentérico;

4) Quantificar marcadores moleculares associados com inflamação e estresse oxidativo;

5) Analisar a atividade de sistemas enzimáticos e não enzimáticos anti-oxidantes.

4. MATERIAIS E MÉTODOS

4.1 Modelo Experimental

Para este trabalho foram utilizados ratos da linhagem Wistar provenientes do biotério do Departamento de Fisiologia e Farmacologia da UFPE, onde receberam ração e água *ad libidum* e foram submetidos a um ciclo de 12 horas claro e 12 horas escuro. No experimento foram utilizados ratos machos vindos de ratas alimentadas com dieta comercial contendo 22% de proteína (Labina®), randomicamente distribuídos, no nascimento, para formar ninhadas de diferentes tamanhos: normal (N6, n=17), composta por 06, e grande (N15, n=20), composta por 15 filhotes. Os animais foram utilizados para o experimento aos 25 dias, logo após o desmame, para análises bioquímicas e morfológicas. Os ratos foram sacrificados por decapitação após overdose de Isoflurano. Os pesos corporais foram obtidos semanalmente até o dia do experimento e o comprimento nasoanal foi medido antes da eutanásia. Apenas os machos foram utilizados para experimentação e todos os experimentos foram feitos com animais de pelo menos 3 ninhadas distintas. Fêmeas permaneceram na ninhada, mas não foram analisadas. Todos os procedimentos foram aprovados pelo Comitê de Ética em Experimentação animal da Universidade Federal Pernambuco (Processo nº 23076.055875/2012-11, Anexo A).

4.2 Preparação do tecido

Aos 25 dias, após decapitação, foi realizada uma laparotomia para obtenção da porção distal do cólon (caracterizado pelo fim do cólon proximal, a partir do desaparecimento das pregas oblíquas da mucosa até cerca de 1 cm do ânus). Após lavagem com PBS (0.15 mol/L NaCl em 0.01 mol/L tampão fosfato de sódio, pH 7.2), o cólon distal foi dividido em dois segmentos: para microdissecação, de modo a obter preparações da camada muscular e outro segmento processado para emblocamento com parafina. Para a análise bioquímica, foi utilizado o cólon distal inteiro. Em resumo, os cólons frescos foram rapidamente removido e lavado com solução salina (NaCl 0,9%) gelada. Para uma melhor dissociação do tecido, o mesmo foi picotado sob superfície gelada e posteriormente mergulhado em nitrogênio líquido para maceração. Após obtenção do conteúdo macerado, a amostra foi armazenada em solução inibidora de protease (tampão Tris / HCl 20 (pH 7,4) com MgCl2 10 mM, CaCl2 0,6 mM, EGTA 0,5 mM, 1 mM de DTT, fluoreto de fenilmetilsulfonilo 1 mM (PMSF), 2 mg / ml de leupeptina e 0,05% de Triton X-100). Os tubos cônicos contendo as amostras foram mantidos

em gelo e submetidos a agitação 6 vezes a cada 5 minutos, com duração de 1 minuto. Posteriormente as amostras foram sonicadas por 10 minutos, "vortexadas" mais uma vez e então centrifugadas a 4ºC durante 15 minutos a 10.000 rpm. O sobrenadante foi pipetado e separado em alíquotas para análises bioquímicas individuais e armazenado a -80°C. A concentração total de proteína no sobrenadante foi determinada pelo ácido bicinconínico (BCA) através de kit de ensaio de proteínas (Pierce, Rockford, IL, EUA), como descrito por protocolo do fabricante.

4.3 Análise bioquímica dos componentes do sangue

Para a análise de colesterol total, triglicerídeo e albumina, o sangue foi coletado na hora da decapitação e colocado em tubo de ensaio. As amostras foram centrifugadas a 3000 rpm durante 15 minutos para obtenção do soro, e foram analisadas através de kit comercial (labtest) com leitura em espectrofotômetro.

4.4 Processamento histológico e análise morfométrica do cólon distal

Um fragmento de aproximadamente 1,0 cm foi utilizado para o estudo histológico. O material foi fixado em paraformaldeído 4% por 24h e seguiu as etapas da técnica histológica convencional. A desidratação iniciou-se com concentrações crescentes de álcool (70%, 80%, 90%, 100% I e 100% II) sendo 1 hora em cada concentração. Seguiram-se a diafanização com xilol (dois banhos de 1 hora) e a impregnação pela parafina, fundida em estufa a 60ºC (dois banhos de 1 hora). Para obtenção do bloco, o tecido foi imerso em um molde retangular que continha parafina fundida e seccionado em micrótomo, obtendo-se cortes de 5 µm. Os cortes foram estirados em água aquecida e dispostos em lâminas, seguindo para desparafinização, hidratação e coloração pela Hematoxilina e Eosina (HE) onde foram analisadas a espessura da *tunica muscularis* e da parede total do cólon distal. A medição de 50 pontos da *tunica muscularis* (10 seções / animal) foi realizada a partir de imagens capturadas com uma objetiva de 10x num microscópio de luz (Nikon Eclipse 50i) acoplado a uma câmera (Nikon DS-Fi1) e analisados com o software Image J (National Institute of Health; versão 1.48).

4.5 Obtenção do plexo mioentérico do cólon distal

Inicialmente o cólon foi removido, lavado com PBS, cortado pela borda mesentérica, esticado e preso em uma superfície lisa e fixado em paraformaldeído 4% por 24 horas a 4ºC. Os intestinos tiveram sua largura mensurada com auxílio de paquímetro para efeito de

comparação entre os grupos. Logo após foram feitas 3 lavagens com PBS e mantidos no mesmo tampão a 4ºC. As camadas do cólon foram dissecadas até a obtenção da camada muscular longitudinal, onde se encontra o plexo mioentérico.

4.6 Imunohistoquímica do plexo mioentérico para evidenciação de célula glial e neurônio entérico.

A análise das células da glia e dos neurônios entéricos foi realizada na sequência da microdissecção, tal como descrito anteriormente. Após bloqueio da peroxidase endógena (TFO 0,1M + 0,05% de H2O2) por 30 minutos e bloqueio das marcações inespecíficas com BSA (BSA 3% em TFO-Triton (0,3% Triton X-100) também por 30 minutos, as amostras de tecido foram incubadas por 12h a 4ºC com o anticorpo anti-GFAP para glia (1:200, Sigma) ou o anticorpo monoclonal anti-HuC/HuD para neurônio (1:500, Invitrogen). Após lavagens com TFO 0,1M, as amostras foram incubadas com o anticorpo secundário biotinilado correspondente, *anti-rabbit* para o GFAP e *anti-mouse* para o HuC/HuD, ambos numa diluição de 1:1000, durante 02 horas. Em seguida o material foi lavado com TFO 0,1M e incubado no complexo ABC (Avidina-Biotina-Peroxidase) por 01 hora, para em seguida ocorrer a reação de revelação com o DAB (3,3' – Diamino Benzidine). Após revelação, o material foi montado em lâmina gelatinizada, desidratado e coberto com lamínula.

4.7 Tamanho do soma dos neurônios do plexo mioentérico do cólon distal.

Para análise do tamanho do soma dos neurônios entéricos, imagens foram capturadas em microscópio óptico na objetiva de 40x e mensurados através do software de análise de imagem, Image J. Foram mensurados o tamanho de pelo menos 100 células aleatórias de cada animal (5 animais por grupo).

4.8 Dosagem da concentração de substâncias reativas ao ácido tiobarbitúrico (TBARS)

A determinação da peroxidação lipídica da membrana foi determinada pela reação formada pelas espécies reativas ao ácido tiobarbitúrico (TBARS). O método foi realizado como descrito em Ohkawa et al. (1979) (OHKAWA; OHISHI; YAGI, 1979), com algumas modificações. Sucintamente, os sobrenadantes dos homogenados do cólon foram colocados em tubos de vidro (200ul) e a reação se deu pela adição de 80 µl de dodecil sulfato de sódio 0,8 %, 600 µl de ácido acético 20 % e 600 µl de ácido tiobarbitúrico 0.8 %. Em seguida os

tubos permaneceram em banho-maria fervente por 1 hora, sendo posteriormente resfriados em água corrente. Após o resfriamento foi acrescentado o álcool butílico com posterior homogeneização e centrifugação a 4.725 rpm por 10 minutos. Por fim, 200 µL do sobrenadante (camada orgânica) foram pipetados em microplaca de 96 poços seguida de leitura em leitor de microplaca a 535nm. Todas as amostras foram feitas em triplicatas e o valor do TBARS foi determinado usando uma curva padrão de derivado de malonaldeído 1,1,3,3,-tetrametoxipropano, por fim os resultados foram normalizados pela concentração de proteína contida em cada homogenado.

4.9 Avaliação da atividade da superóxido dismutase total (tSOD)

A atividade enzimática da SOD foi determinada segundo Misra & Fridovich, (1972) (MISRA; FRIDOVICH, 1972) à temperatura ambiente. Foram utilizados 60 µl do sobrenadante foram adicionados a 920 µL de tampão carbonato de sódio (0,05%, PH 10.2, 0,1mM de EDTA). Para iniciar a reação adicionou-se 20 µL de epinefrina 30 mM (dissolvido em solução de ácido acético 0,5%) à mistura. A leitura foi realizada com cubeta de quartzo em espectrofotômetro através de 480nm. As leituras foram realizadas a cada 15 segundos durante 150 segundos. A atividade enzimática foi expressa como a média de valores adquiridos quando ocorreu 50% de inibição da oxidação da epinefrina que equivale a 1unidade. As leituras foram realizadas em triplicatas e expressas em U/mg de proteína.

4.10 Avaliação da atividade da Catalase (CAT)

A atividade da catalase foi avaliada segundo o método descrito em Aebi, (1984) (AEBI, 1984), utilizando H_2O_2 como substrato. Brevemente, 60ul do sobrenadante foram adicionados a 905ul de tampão fosfato (50 mM pH 7,0; 37ºC) e 35ul de peróxido de hidrogênio (300 mM). A leitura foi realizada com cubeta de quartzo em espectrofotômetro através de um comprimento de onda de 240 nm. As leituras foram realizadas a cada 12 segundos durante 120 segundos. As leituras foram realizadas em triplicatas e expressas em U/mg de proteína.

4.11 Níveis de Glutationa reduzida (GSH)

Os níveis de GSH foram analisados segundo o método de HISSIN e HILF (1976) (PAUL; HILF, 1976). Foram adicionados 1,8 mL de tampão fosfato-EDTA (pH = 8.0) mais 100 µL de solução de ortoftaldeído (OPT) utilizados a 100 µL do sobrenadante, obtendo-se

assim, uma solução final de 2 mL. Esta solução foi incubada por 20 minutos à temperatura ambiente e protegida da luz. Logo depois foi feita leitura em espectrofluorímetro utilizando o comprimento de onda de 350 ηm para emissão. Previamente, foi preparada uma curva padrão com concentrações de GHS entre 0,01 – 1 mM.

4.12 Níveis de Glutationa oxidada (GSSG)

Os níveis de GSSG foram analisados segundo o método de HISSIN e HILF (1976)(PAUL; HILF, 1976). Brevemente, 0,5 mL do sobrenadante de homogenado do cólon foi incubado à temperatura ambiente com 200 µL de NEM 0.04 M (N-etilmaleimide) por 30 minutos para interagir com a GSH presente no tecido. Para esta mistura, 4,3 mL de NaOH 0.1 N foi adicionado. Para medir os níveis de GSSG, 100 µL desta mistura foi utilizada usando o mesmo procedimento descrito para o ensaio de GSH, exceto 0.1 N NaOH , que será utilizado como diluente ao invés de tampão fosfato-EDTA.

4.13 Níveis de óxido nítrico no cólon (NO)

A concentração de nitrito foi estimada utilizando o reagente de Griess (Sigma), que serve como indicador da produção de óxido nítrico como descrito por Green et al. (1982) (GREEN et al., 1982). Volumes iguais do sobrenadante e do reagente (100 µl) foram colocados em placas de 96 poços e reagiram por 10 minutos em temperatura ambiente. As absorbâncias foram lidas no comprimento de 540 nm. O experimento foi realizado em triplicatas e o resultado foi expresso em µmol de nitrito por mg de proteína em referência a uma curva padrão construída com concentrações conhecidas de nitrato de sódio.

4.14 Quantificação de citocinas

Foi realizada a quantificação de 3 citocinas a partir dos sobrenadantes de homogenados do cólon: Ilterleucina-1beta (IL-1β), IL-10 e TNFα (fator de necrose tumoral alfa). Para tal, foi utilizado o método de ELISA (Enzyme Linked Immuno Sorbent Assay) através de kits específicos (eBioscience) e seguindo as recomendações do fabricante. A leitura das amostras foi realizada em espectofotômetro (562 nm) e a quantidade de citocina em cada amostra foi deteminada pela relação do valor de citocina encontrado pelo ensaio enzimático sobre o valor total de proteína no tecido (pg de citocina / mg de proteina).

4.15 Análise estatística

A análise estatística foi realizada através do Graphpad Prism 5.0. Para determinação de normalidade da amostra foram utilizados os testes de normalidade Kolmogorov-Smirnov e Shapiro-Wilk. O teste t-student não-pareado foi utilizado para comparação entre os grupos controle e experimental para os dados paramétricos, onde os dados foram expressos com média $\pm$ desvio padrão e o teste Mann-Withney para os dados não paramétricos, onde os dados foram expressos como mediana $\pm$ inter-quartis. Diferenças entre grupos foram consideradas estatisticamente significativas quando $p \leq 0,05$.

5. Capítulo 1

Artigo na forma de manuscrito a ser submetido na revista

"Neurogastroenterology & Motility"

Malnutrition induced by larger litters increase NO production and induces inflammatory status without modify antioxidant resource in the distal colon of lactating rats

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Abstract

Background Nutrients and trophic factors of breast milk are crucial to enteric nervous system development, especially to maintain short and long-term gastrointestinal functions. Nevertheless, the repercussion of low amount of feeding during suckling on large intestine anti-inflammatory and anti-oxidant resources, especially those involving myenteric glial cells has not yet been completely understood. This study hypothesized that unfavorable lactation is able to induce oxidative stress and inflammatory conditions in the colon of weanling rats. **Methods** Wistar rats were reared in different early nutritional conditions according to litter size in two groups: N6 (6 pups/dam) and N15 (15 pups/dam) until the 25th post-natal day. Under isofluorane anesthesia, the distal colon was removed and processed for biochemical, morphometric and immunohistochemical analyzes. Homogenates of distal colon were analyzed for lipoperoxidation, nitric oxide (NO), GSH, GSSG, TNF-alfa, IL-1β and IL-10 levels, and total superoxide dismutase (tSOD) and catalase (CAT) activities. Morphometric analysis was carried out using paraffin sections and longitudinal muscle and myenteric plexus preparations. **Key Results** Increased levels of lipoperoxidation, NO, TNFa, IL1b and CAT, but not tSOD, were found in the N15 compared to N6 group. No intergroup difference was detected in GSH and IL10 levels but GSH/GSSG ratio was higher in the N15 group. Lower size of myenteric neurons, and increased density of lymphocytes were found in the N15 group. **Conclusions & Inferences** Reduced feeding during suckling induces inflammatory and oxidative status in the colon of weanling rats. The data suggest potential mechanisms by which malnutrition early in life can induce vulnerability of distal colon to adverse stimuli increasing the risk of long term gut diseases.

Keywords: Large litters, Enteric nervous system, Myenteric glial cells, Myenteric plexus, Myenteric Neurons, Nitric oxide, Gut inflammation.

Introduction

As an autonomic division of the peripheral nervous system, the enteric nervous system (ENS) plays crucial roles in the gastrointestinal functions, modulating motility, secretion, immune responses and maintenance of the intestinal epithelium(1,2). ENS development depends on a coordinated migration, expansion and differentiation of progenitor cells of the neural crest (NC) from the vagal and sacral regions of the neural tube during embryogenesis (3). During migration, NC progenitor cells proliferate and give rise to neurons and glial cells, colonizing the entire length of the gut by embryonic day 14 in the mouse and week 7,5 in humans(2,4–6). Most neurons and glial cells are condensed into myenteric and submucosal ganglia defining an intricate network of several cell subclasses (2,7). In addition to intraganglionic location, enteric glial cells are also associated with nerves within the circular musculature and the mucosa underlying the epithelium. Mucosal glial cells are able to integrate intercellular signaling, connecting immune and enteroendocrine cells in addition to blood vessels (8). The establishment of functional circuits of the ENS occurs only after birth, mainly during the first month of life in rodents (8,9).

Interactions among the ENS, smooth muscle and lumen of the gut are involved in effective development and plasticity of the gastrointestinal functions (10). In this aspect, adequate breast-feeding and microbiota are fundamental players to allow short and long-term changes and adaptive responses of neurons and glial cells (11). Enteric glial cells are crucial for the maintenance of gut homeostasis, releasing trophic factors and actively regulating the redox balance and inflammatory response against pathogens (12) as well as the integrity of the gut epithelium, protecting the intestinal barrier(13,14). They are recognized as immunecompetent cells; express molecules such as substance P, which can activate mast cells and macrophages (15) and the S100B protein, involved in signal activation of gut inflammatory processes (16,17). Ablation of enteric glia in the small intestine of adult transgenic mice induce fulminating jejunoileitis with severe inflammation and hemorrhagic necrosis of this organ (18) .

Enteric neurons are involved in the proliferation of gut epithelial cells and express membrane transporters and receptors involved in nutrient detection (19). During lactation period, trophic factors present in the maternal milk are crucial to neuronal survival and differentiation (20).

Epidemiological studies have correlated undernutrition with weakened gastrointestinal function, impaired innate and adaptive host immune responses, disrupted intestinal barrier (21,22), increased probability of diarrhea (23) and recently with defects in the infant gut microbiota maturation (24). Some studies have indicated the lack of breast feeding as a risk factor associated with later development of Crohn's disease in the colon (25–28). However, the repercussion of low amount of feeding during lactation on the large intestine and especially on enteric glial cells has not yet been completely understood. Bearing in mind the importance of the suckling period for an adequate development of the ENS the present study hypothesized that unfavorable lactation is able to impair the structure and function of enteric neurons and glial cells, inducing oxidative stress and inflammatory status in the colon of lactating rats. To address this question it was adopted the experimental model of under nutrition by manipulation of size litters only during lactation period in order to discard any interference of gestational nutritional condition of the mothers. This experimental model has been regarded as a naturally varying feature of the early developmental environment and may induce permanent modifications in the brain innate immune system of adult rats (29,30)

METHODS

Animals and lactation condition

All procedures applied to this investigation were submitted to and approved by the institutional animal care committee of the Federal University of Pernambuco, Brazil (Approval Protocol no. 23076.055875/2012-11), in accordance with the ‘‘Principles of Laboratory Animal Care’’ (National Institute of Health, NIH). Wistar rats were kept in an environmentally controlled room at 25 ± 2◦C with a light/dark cycle (lights on 06:00h). Male wistar newborn rats from dams fed with a commercial rat chow diet containing 22% of protein (Labina®, Purina do Brasil Ltd, São Paulo, Brazil) were randomly distributed at birth into different early nutritional conditions and divided according to litter size in two groups: Control (N6, 6 pups/dam, n= 17) and undernourished (N15,15 pups/dam, n= 20) until the 25th postnatal day, when they were sacrificed for experimental procedures. Only male offspring were used for experimentation and all experiments were carried out with animals of at least 3 different

litters. Females remained in the litters but were not investigated. The body weights of the animals were recorded every week until sacrifice.

Tissue collection

At 25 days, the animals were sacrificed under overdose of Isofluorane and the nasoanal length was measured. A laparotomy was performed to collect the entire large intestine and samples of the distal colon (characterized by the end of the proximal colon, starting from the disappearance of mucosal oblique folds until approximately 1 cm from anus, supplementary figure). After washing with PBS (0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2), the distal colon was divided into two segments: one for microdissection, to obtain wholemount preparations of the muscular layer and the other segment to be processed for paraffin embeddeding. For biochemical analysis, the total distal colon was used. Briefly, the freshly dissected colons were immediately frozen in liquid nitrogen to macerate and obtain the homogenate containing a cocktail of protease inhibitors (20 mM Tris/HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.5 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml leupeptin and 0.05% Triton X-100). After this, the homogenized samples were centrifuged at 10,000 rpm for 15min at 4∘C. The supernatant was separated into different aliquots for individual biochemical estimations and stored at−80°C. Total protein concentration of the supernatant samples was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) as described by the manufacturer's protocol.

Biochemical analysis of blood components

To analyze total cholesterol, triglycerides and albumin, blood was collected and placed in test tube. The samples were centrifuged at 3000 rpm for 15 minutes to obtain serum, and were analyzed using an commercial kit (Labtest).

Histological preparation and examination of distal colonic sections

After an overnight fixation with 4% paraformaldehyde, 1.0 cm section of distal colon was dehydrated in an ascending series of ethanol (80%, 90%, and 100%), cleared in xylene, and embedded in paraffin to obtain 5 μm thick sections using a microtome, which were stained with hematoxylin and eosin. The measurement of 50 points of the

tunica muscularis (10 sections/animal) was performed from images captured with a 10x objective on an light microscope (Nikon Eclipse 50i) coupled to a camera (Nikon DS-Fi1) and analyzed with Image J software (National Institutes of Health; version 1.48).

Preparations of the wholemounts and immunohistochemistry of myenteric neurons and glial cells

Fresh segments of distal colon were removed and placed in PBS. The dissected pieces were opened along the mesenteric border and cleaned of their contents using PBS. They were then pinned, mucosa-side down, onto a balsa isopor board and fixed overnight at 4℃ in paraformaldehyde 4%. The next day, the tissue was measured with pachymeter, in order to obtain the width of distal colon. Wholemount preparations were obtained by removing the mucosa, submucosa and circular layers, using a stereomicroscope. The remaining two wholemounts from each animal were subjected to immunohistochemical staining with HuC/HuD and GFAP to evaluate the neuronal and enteric glia subpopulations, respectively. Briefly, wholemounts were rinsed in PBS, soaked for 30 min in 3% H2O2 (to block endogenous peroxidase), rinsed in PBS, and placed for 30 min in 3% bovine serum albumin and 0.3% Triton X-100 in PBS (to block nonspecific protein). The preparations were then exposed overnight (at 4ºC) to mouse anti-HuC/D (1:500; Molecular Probes) and rabbit anti-GFAP (1:200, Sigma) in 1% bovine serum albumin and 0.3% Triton X-100 in PBS. Wholemounts were rinsed in PBS, incubated for 1h with the respective secondary antibodies (1:1000, Jackson Immunoresearch Laboratories), incubated for 1 h in avidin biotinylated peroxidase complex (1:200, ABC Elite kit; Vector Laboratories), rinsed in PBS, and reacted for 5 min in 3,3'-diaminobenzidine (DAB; 0.07%) and H2O2 in PBS. Stained preparations were rinsed in distilled water, mounted on gelatin-coated slides, air-dried overnight, dehydrated in ethanol, cleared in xylene, and coverslipped with entellan (Merck).

Analysis of the myenteric plexus

For the qualitative analysis of the organizational structure of the myenteric plexus, by immunostaining for GFAP and the morphometric study of neurons, images were captured with a 40x objective on a light microscope (Nikon eclipse 50i) coupled to a camera (Nikon DS-Fi1). In the images obtained, were measured the profile of 100

neurons per animal (5 per group) using the software Image J (National Institutes of Health; version 1.48).

Assay of Lipid Peroxidation

Lipid peroxidation was measured by estimation of malondialdehyde (MDA) by thiobarbituric acid (TBA) reaction (TBARS method) according to Ohkawa et al. (1979) (31), with some modifications. In TBA test reaction, MDA or MDA-like substances and TBA react to produce a pink pigment with maximum absorption at 532 nm. The reaction was developed by the sequential addition of 200 µL of colonic homogenates, 80 µL of 0,8% sodium duodecil sulfate, 600 µL of 20% acetic acid (pH 3.5) and 600 µL of 0.8% TBA solutions in a boiling water-bath for 60 min to triplicates of supernatants. After tap water cooling, 1.5 mL of n-buthanol / pyridine (15:1 v/v) was added to the sample, centrifuged at 2,500 g for 10 min and the absorbance of the organic phase (200 µL) was read at 532 nm in a plate reader. Results were expressed as nmol per mg of protein using a standard curve of 1,1,3,3-tetramethoxypropane solution. The results were expressed in nmol MDA /mg protein. Control samples were incubated in a 30 µM sodium nitroprusside solution for 45 min before the assay and used as positive controls for lipid peroxidation.

Superoxide Dismutase (SOD) Assay

Total SOD (t-SOD) enzymatic activity was performed according to Misra and Fridovich, (1972) (32) at 25°C. Triplicates of supernatant of colonic tissue homogenate (60 µL) were previously incubated in a water bath at 37°C and then added to 920 µL of 0.05% sodium carbonate solution pH 10.2 in 0.1 mM EDTA. The reaction was developed by addition of 20 µL of 30 mM epinephrine (in 0.05% acetic acid). The absorbance was measured at 480 nm for 150 s using quartz cuvette in spectrophotometer. One unit of t-SOD was defined as the enzyme amount causing 50% inhibition of epinephrine oxidation. Tissue t-SOD enzymatic activity was also expressed as units per milligram of protein (U/ mg protein). Positive controls were obtained incubating control homogenate samples in a 30 µM SNP solution for 45 min before the enzymatic assay.

Catalase (CAT) Assay

Catalase activity was measured according to Aebi (1984) (33), using H_2O_2 as substrate. Briefly, 60 µL of supernatant of colonic tissue homogenate were added to 905 µL phosphate buffer (50 mM, pH 7,4) and 35 µL of H_2O_2 (300 mM). The absorbance was measured at 240 nm for 120 s using quartz cuvette in spectrophotometer. The catalase activity was expressed as U / mg protein, and U of enzyme activity, was expressed as consumed H_2O_2 in nM/min/mg protein. Positive controls for catalase activity were initially obtained by incubation of supernatant tissue homogenates of control group in increasing concentrations of H_2O_2 (3.156 to 100µM) for 30 min at 37°C before the enzymatic assay.

Gluthathione assessment

The assessment of levels of reduced (GSH) and oxidized (GSSG) gluthatione in the supernatant of colonic tissue homogenate was done by the method of HISSIN and HILF (1976) (34). For GSH, were added 1.8 ml of phosphate-EDTA buffer (pH 8.0) plus 100 uL of o-phthalaldehyde (OPT) solution in 100 uL of the supernatant, obtaining thus, a final 2 mL of solution. This solution was incubated for 20 minutes at room temperature and protected from light. Immediately after reading was done in spectrofluorimeter using a wavelength of 350 ηm. Previously, a standard curve was prepared with concentrations ranging GSH 0.01 to 1 mM. For GSSG, 500 ul of the supernatant of the homogenate was incubated at room temperature with 200 uL of N ethylmaleimide (NEM) 0.04 M for 30 minutes to interact with the GSH present in the tissue. To this mixture, 4.3 mL of NaOH 0.1 N was added. To measure the levels of GSSG, 100 uL of this mixture was used using the same procedure described for assay of GSH, except that 0.1 N NaOH was used as diluent instead of phosphate-EDTA buffer. The results were expressed in µM/mg protein.

Nitrite assay

The nitrite present in the supernatant of colonic tissue homogenate was used as an indicator of NO production using the Griess reaction like described by Green et al. (1982) (35) . Briefly, the samples (100 µL) were mixed with equal volume of Griess reagent in a 96-well plate and were incubated at room temperature for 10 min. The absorbance was read at 540 nm using a microplate reader and the nitrite concentrations

were determined by comparison with a standard curve of sodium nitrite. The results were expressed in μmol/mg protein.

Measurement of cytokines

The concentrations of TNF-α, IL-1β and IL-10 was measured using sandwich ELISA kits specific for rats (eBioscience, San Diego, California, USA) according to the manufacturer's instruction. Results were expressed at pg/mg protein.

Statistical analysis

All data analysis was performed using GraphPad Prism software (Prism 5.01; GraphPad Software, La Jolla, CA, USA). Normally distributed data are presented as the mean ± standard deviation (mean ± SD). Unpaired t-tests were used to compare parametric continuous variables. Mann-Whitney U test was used to compare data sets of non-parametric data as determined by a D'Agostino-Pearson normality test, and the data are presented as the median ± interquartiles. Level of $P < 0.05$ was considered statistically significant.

RESULTS

Somatic indicators of experimental undernutrition

Body weights of N6 and N15 groups are compared in the Fig. 1A. As can be seen, a significant body weight deficit was detected just at the first (11 %; $p < 0,0001$) and 7th (23 %; $p < 0,0001$) days after birth in the N15 group as compared to control. This deficit increased up to 41% at weaning when nasoanal length (Fig. 1C) was ~18 % lower (13,41 ± 0,44 cm; in N6 and 11,00 ± 0,42 cm in N15 group, $p < 0,0001$) in the N15 group. The body mass index (BMI = body weight (g) / legnht² (cm²) ; Fig. 1D) was ~13% higher in the control group when ccompared with the experimental one (0,355 ± 0,004 g/cm^2; in N6 vs 0,311 ± 0,002 g/cm² in the N15 group, $p < 0,0001$). For all somatic indicators, N6: n=17 and N15: n=20.

Biochemical analysis of blood components

The biochemical analysis of blood components is presented in Fig. 2, no intergroup difference was observed in the levels of total cholesterol (63,73 ± 2,30 mg/dl in N6 and 66,14 ± 4,48 mg/dl in N15 groups; p= 0,60), triglycerides (70,10 ± 5,79 mg/dl in N6 and 74,92 ± 5,96 mg/dl in N15 groups; p= 0,59) and albumin (2,60 ± 0,44 g/dl in N6 and 2,46 ± 0,47 g/dl in N15 groups; p= 0,38). For all biochemical analysis, n=10 per group.

Morphometric parameters of distal colon are modified by undernutrition during lactation

The length of the distal colon in the N15 group was ~15% lower when compared to the control one. Fig. 3A. shows this difference illustrating a representative distal colon of each group. Morphometric analysis of longitudinal preparations showed lower average of distal colon width (~15%) in undernourished group compared to control (1,27 ± 0,09 cm in N6 vs 1,09 ± 0,08 cm in the N15 group; p= 0,001; Fig. 3B).. Thickness of total colonic wall from the outer limit of *tunica muscularis* until the inner border of the *tunica mucosa* in the N15 group was significantly lower, approximately 11% than that of N6 group (1010,05 ± 114,70 µm in N6 and 905,75 ± 169,77 µm in N15 groups, p= 0,001). Regarding the *tunica muscularis* thickness, intergroup difference was about 12% (84,00 ± 22,30 µm in N6 and 74,02 ± 22,38 µm in N15

groups, p= 0,006) (Fig. 3C). Images of paraffin embedding coronal sections stained by HE are shown in the Fig. 3D. For all morphometric analysis, n=5 per group.

Longitudinal preparations show preserved glial network in the myenteric plexus but smaller neuronal cell bodies in undernourished rats during lactation

GFAP imunorreactivity did not show apparent structural disorganization of myenteric plexus in the N15 group (Fig. 4). Nevertheless, morphometric analysis of myenteric neurons, specifically stained by HuC/HuD (Fig. 5A), demonstrated a different soma size profile between the groups. The soma size frequencies of the predominant classes of neurons within the myenteric plexus of each group were found to be distinguishable from a normal distribution (Shapiro Wilk's W test, p<0,0001; Fig. 5B). Median of soma area differed significantly between the groups showing smaller cells in undernourished animals (N6: median 310,85; min 77,96; max 987,37 and N15: median 291,74; min 114,68; max 993,41 μm^2; Mann Whitney test, p =0,026; Fig. 5C; n=5 per group).

Oxidative status of distal colon is modified by undernutrition during lactation

Biochemical results related to lipoperoxidation and antioxidant markers are summarized in Fig. 6. TBARS levels were 2.5 fold greater in the distal colon of N15 group (2,51 ± 0,22 nmol MDA/mg protein) compared to control condition (1,079 ± 0,10 nmol MDA/mg protein; Fig. 6A). A significantly lower (~36%) of tSOD (Fig. 6B) enzyme activity was found in the undernourished animals compared to the control group (N15; 0,056 ± 0.018 U/mg protein and N6; 0,088 ± 0,023 U/mg protein, p<0,001). The reverse was observed when comparing CAT activity (Fig. 6C), a significantly higher activity (~60%) was observed in the undernourished animals when compared to the control (N15; 0,049 ± 0,024 U/mg protein and N6; 0,027 ± 0,010 U/mg protein, p<0,001). Intergroup difference was not observed for GSH levels (60, 89 ± 21, 26 µM/mg protein in N6 and 53,34 ± 26,19 µM/mg protein in N15 groups). On the other hand, lower levels of GSSG were detected in the N15 group (14,41 ± 4,12 µM/mg protein vs 27,48 ± 4,59 µM/mg protein in the control) increasing GSH/GSSG ratio (2,32 ± 0,33 vs 3,58 ± 0,53 in the control; Fig. 6D). n= 7 per group

Moderate inflammatory status is induced in the colon of undernourished animals

Fig. 7 shows the results obtained for NO, TNF-α, Il-1β and IL-10 levels in homogenates of colon of both groups. NO production assessed by nitrite levels (Fig. 7A) was 1.5 fold greater in N15 group (4,85 ± 1,85 µmol/mg protein) as compared to control (3,04 ± 0,63 µmol/mg protein). TNF-α (Fig. 7B) and IL 1β (Fig. 7C) levels were also increased by undernutrition about 20 and 25%, respectively (1,57 ± 0,24 pg/mg protein vs 1,26 ± 0,26 pg/mg protein in the control, p= 0,03) and (1,17 ± 0,13 vs 0,88 ± 0,15 pg/mg protein in the control, p= 0,02). However, no intergroup difference was observed in the levels of IL-10 (51,22 ± 8,15 pg/mg protein in N15 and 44,54 ± 13,09 pg/mg protein in N6 groups, p= 0,26; Fig. 7 D).

Structural evidence of gut inflammation in the colon

Qualitative microscopic analysis of the distal colon using HE stained cross sections indicated an apparent higher density of lymphocyte in the mucosa layer (Fig. 8) and increasing muscular layer of mucosa (Fig. 8, arrowhead). Inserts shows higher magnification images illustrating the presence of lymphocytes in representative sections of both groups.

DISCUSSION

It is well established in the literature the importance of breastfeeding in preventing age-related diseases affecting the gastrointestinal tract during the newborn period (28,36). Agents in the maternal milk change the gut physiologic state adapting this system in the transition of intrauterine to extrauterine lifes, promoting the growth of intestinal villi, the changes in the permeability of the gastrointestinal tract and resistance to certain inflammatory insults and immune-mediated diseases (37). On the other hand, malnutrition early in life has been associated to higher probability to develop diarrhea in newborns and inflammatory diseases in adulthood especially those involving the large intestine (20,23)

Considering the importance of maternal milk nutrients to an adequate development of the ENS (11,20), the present study hypothesized that reducing feeding during suckling is able to impair the structure and function of enteric neurons and glial cells, inducing oxidative stress and inflammatory status in the colon of weanling rats.
The results partially support our hypothesis, demonstrating that lipoperoxidation, NO production and pro-inflammatory markers such as TNF-α and IL-1β were increased by this nutritional insult. Imbalance in the antioxidant enzymes was also detected. On the other hand, IL-10 and GSH were not modified and surprisingly GSH/ GSSG ratio was increased.

As has been previously reported, the manipulation of rat litter size during suckling was able to induce systemic effects on the rat somatic growth as shown by the reduced body weight (38), nasoanal and intestinal length, detected in the weanling animals. The deficit of ~41 % in the body weight of N15 group as compared to the N6 animals represent the maximum limit of a moderate malnutrition status according Gordon et al., 2012 (24). In addition to a limited number of suckling opportunities per pup, it cannot be discarded that a reduced maternal care in a litter containing 15 pups /dam can also interfere in the body weight gain during lactation period. It is well known the importance of skin-to skin contact between the mother and newborns as an adequate stimulus for somatic and affective development (39). Despite the modifications, in the somatic growth, no changes in the serum albumin, cholesterol, and triglycerides levels were found in the weanling malnourished group suggesting the absence of severe systemic metabolic changes. These results seems to be coherent with the fact that

despite the low amount of feeding there is not lack of specific nutrients in the maternal milk. It is possible that the adequate nutritional condition of the mothers during gestation favored the quality of the milk provided to the newborns.

Usually the structure of large intestine has been considered more resistant than the small one under different types of environment insults (40). Protein malnutrition experimental studies on rats of different ages showed that the small intestine presents reduction of the intestinal wall layer thickness (41,42) as well as the reduction of the thickness of the mucosa tunic and enterocyte height (43). In the colon of adult animals, a few number of studies also demonstrated reduced total wall and tunica mucosa thickness when malnutrition was induced by protein-restricted diet (44–46). In the present study, reduced feeding during lactation was also able to induce structural modifications in the distal colon especially characterized by a smaller perimeter of the transversal section (~15%) and thickness of total colonic wall (11%) and *tunica muscularis* (12%). These findings in the colon of weanling rats differ from those reported for animals submitted to dietary restriction in adulthood (47). Where no structural effect of malnutrition was found in the large intestine. Considering the importance of the gut for the protein turnover and energy expenditure of the entire body, it is possible that structural modifications in the large intestine, herein detected represents also an adaptive response of a smaller organ to a smaller food intake as previously stated by Deo (1978) (48).

An apparent preservation of myenteric plexus immunoreacted by GFAP was herein detected in the distal colon of N15 group while modifications in the neuronal soma size profile were found. These results in weanling rats reinforce the relative vulnerability of the neuronal cells to nutritional deficiency. According to previous studies, protein malnutrition affected the neuronal growth in the large intestine either when is installed in adulthood (45) or when is chronically imposed from gestation until 42 days of life (49) or yet until adulthood (50). Myenteric neurons display a wide range of sizes (100µm² e 1000µm²), which corresponds to several cell subtypes regarding function. During lactation period, trophic factors present in the maternal milk are crucial to neuronal survival and differentiation (20). Enteric neurons are involved in the proliferation of gut epithelial cells and express membrane transporters and receptors involved in nutrient detection (19)..Therefore, modifications in their size at condition of reduced suckling may represent not only an adaptive condition but also can impair the

ENS function, especially considering recent evidence that the presence or absence of nutrients in the intestinal lumen induces long-term changes in neurotransmitter expression, excitability, neuronal survival and has some impact upon gut motility, secretion or intestinal permeability (11).

Despite the apparent preservation in the distribution of GFAP positive enteric glial cells (EGC) in the myenteric plexus of N15 group, we cannot discard modifications in the functional activity of these cells. It is recently recognized that EGC play important roles in inflammatory and oxidative status of the gastrointestinal tract (51). EGC have receptors for a number of pro-inflammatory citokines such as IL-1, IL-6 (15,52,53) and can inhibit inflammation in animal models of colitis for example, secreting nerve growth factor neurotrophin-3 and glial derived neurotrophic factor (GDNF) prevents also cell apoptosis (54,55). In the present study, the higher levels of NO (~1.5 fold), TNF-α,(~20%) IL-1β (~15%) associated to increased lipoperoxidation and impaired function of tSOD activity found in the malnourished weanling animals indicates a moderate inflammatory status and a reduced enzymatic anti-oxidant resource.

Interleukin IL-1β and TNF-α are usually released under pathological conditions in the gastrointestinal tract such as inflammatory bowel diseases. While EGC proliferation can induce protective mechanisms, in a dose dependent manner, IL-1β can inhibit this proliferation (56). In contrast, the anti-inflammatory interleukin IL-10 is able to induce a biphasic response; and in higher concentrations stimulates EGC proliferation (56). IL-10 can also modulate TNF-α levels and its depletion causes a spontaneous development of gut transmural inflammation (57). During lactation, IL-10 is provided in expressive amount by the maternal milk (58). No intergroup difference was found in the levels of this interleukin in the colon of N15 group, which suggest some level of protection in spite of the state of malnutrition.

In favor to this hypothesis no significant difference was detected in the GSH levels in the distal colon of N15 group when compared to N6 animals. It has been established that the synthesis of GSH by EGCs can minimize effects of oxidative stress on neuronal damage (59). In the present study the preservation of GSH levels was also accompanied by surprisingly lower levels of GSSG, indicating a positive redox balance regarding the activity of this non enzymatic antioxidant system. Although mechanisms involved with lower levels of GSSG are not clear at this moment, and deserves future

studies, the apparent preservation of GSH levels in the colon seems to indicate that EGC of the malnourished group are not completely affected.

Oxidative stress can be also involved in the etiology of intestinal inflammatory condition (60). Higher levels of lipoperoxidation herein detected in the malnourished group were apparently associated to an impaired tSOD activity and increased NO production. The constitutive production of NO is important to maintain protective actions on the gastrotintestinal mucosa especially due to its action on the blood vessels and a modulatory effect on mast cells (61). However, an excessive amount of NO in condition of gut inflammation can induce deleterious effects on the epithelial permeability in the mucosa (62). Increases of 2 – 5 fold in the NO levels were detected in humans with inflammatory colon diseases (63). The significant increase of ~40% found in the colon of malnourished weanling rats was accompanied by qualitative changes in the structure of colon as indicated by a higher density of lymphocytes in the mucosa. Analysis of the reactive state of these lymphocytes as well as potential mechanisms involved in the NO production are under investigation in our laboratory at this moment.

Conclusion

The present findings reinforce the importance of a favorable condition of lactation for healthy development of the enteric nervous system and colon in neonates. Taken together, the data suggest potential mechanisms by which modifications in the anti-oxidative and anti-inflammatory condition of the large intestine early in life can induce vulnerability of distal colon to adverse stimuli. It is possible that these mechanisms can increase the risk of long term gut diseases.

Acknowledgments, Funding, and Disclosures

• **Eraldo Fonseca dos Santos Júnior:** Performed the research, designed the research study, analyzed the data and wrote the paper.

• **Catarina Gonçalves Pimentel:** Designed the research study and analyzed the data

• **Larissa Cardoso Correia de Araújo:** Contributed with essential reagents and tools for measurement of cytokines.

• **Teresinha Gonçalves da Silva:** Contributed with essential reagents and tools for measurement of cytokines.

• **VivaldoMoura-Neto:** Analyzed the data

• **Belmira Lara da Silveira Andrade-da-Costa:** Designed the research study, analyzed the data and wrote the paper

We are also grateful to Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) which provided financial support and scholarships for Eraldo Fonseca dos Santos Junior and Catarina Gonçalves-Pimentel (DCR 0079-2.07/10)

There is no conflict of interest in the work reported in the present paper.

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Figure 1

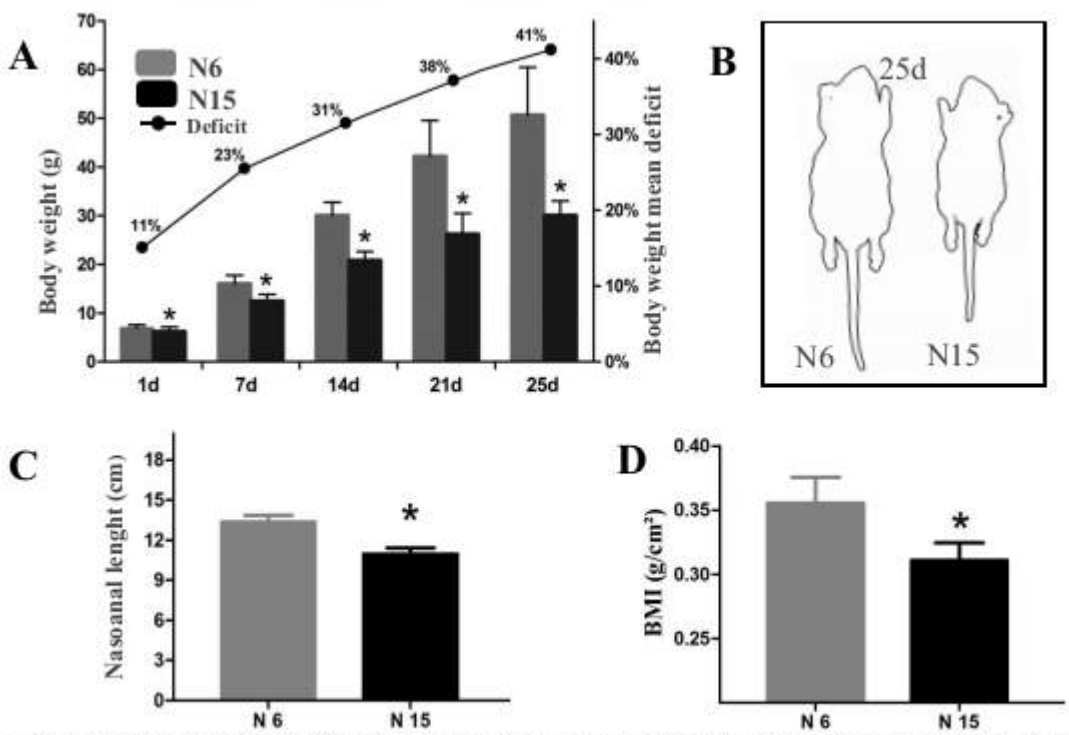


Figure 1 Somatic indicators of experimental undernutrition. The mean body weight (left Y-axis) is shown over time for control (N6) and undernourished rats (N15). The right Y-axis indicates the percent deficit in body weights between the two groups (dotted line) (A). Representative figure of rats at 25 days of N6 and N15 group (B). Nasoanal length (C) and body mass index (BMI) (D). N6; n= 17 and N15; n=20. Unpaired t test, * $p < 0{,}0001$. Data are presented as mean ± SD

Figure 2

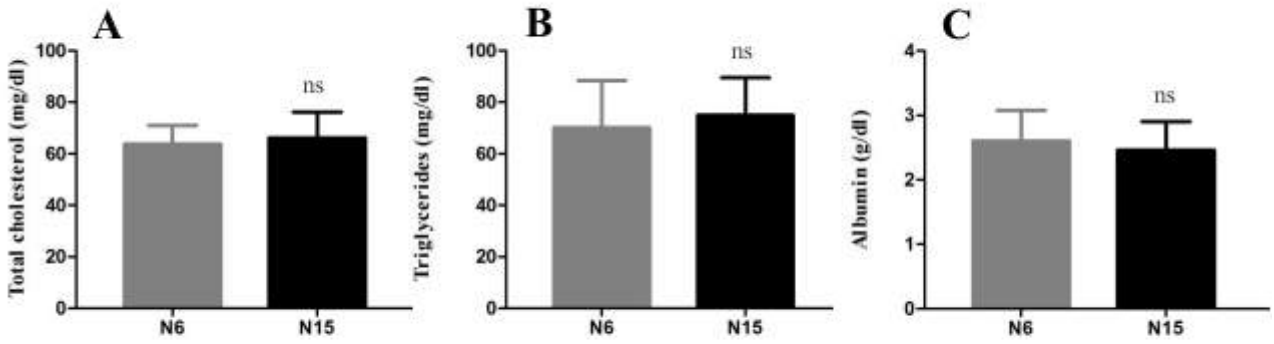


Figure 2 Effects of litter size on blood components. Levels of total cholesterol (A), triglycerides (B) and albumin (C) in control (N6) and undernourished (N15) rats at 25 days. n=10 per group. Unpaired t test. ns, no significance.
Data are presented as mean ± SD

Figure 3

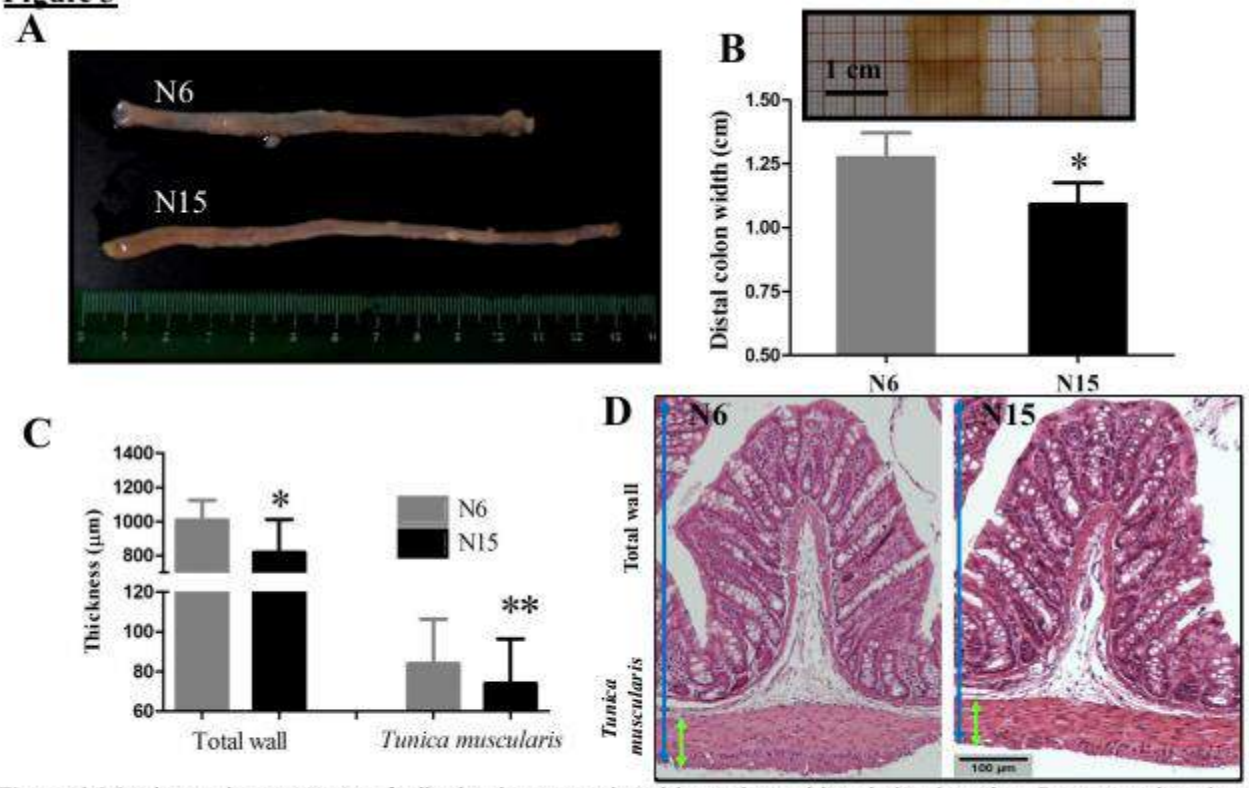


Figure 3 Morphometric parameters of distal colon were altered by undernutrition during lactation. Representative photo comparing the length of the distal colon of nourished (N6) and undernourished animals (N15) (A). Morphometric analysis of distal colon width (B) and thickness of total wall and *tunica muscularis* between groups (C).Representative images of cross-sections of distal colon stained with hematoxylin and eosin, showing total wall (blue arrow) and *tunica muscularis* (green arrow) (D). n=5 per group, Unpaired t test, * p = 0,001, ** p = 0,006. Data are presented as mean + SD

Figure 4

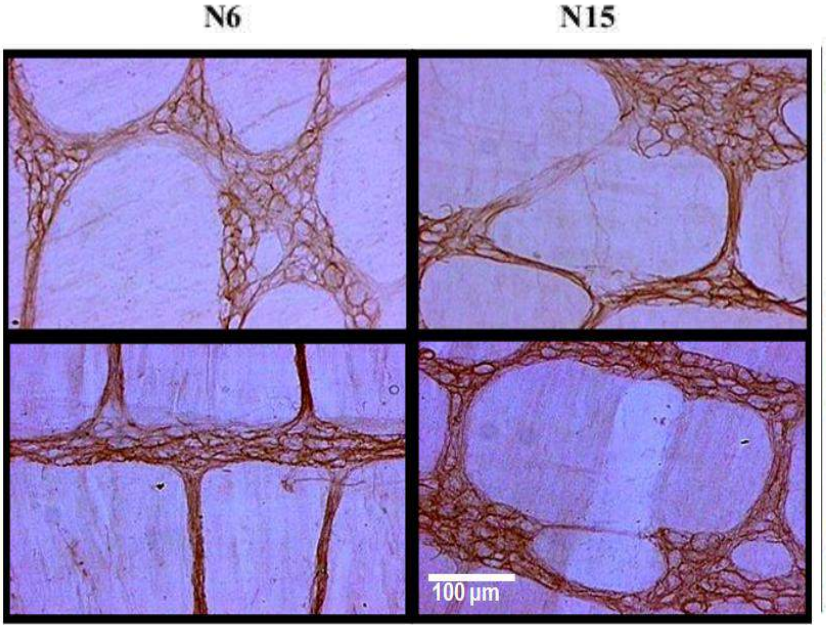


Figure 4 Representative photomicrographs of immunostaining for GFAP (glial cell) in the myenteric plexus of the distal colon.

Figure 5

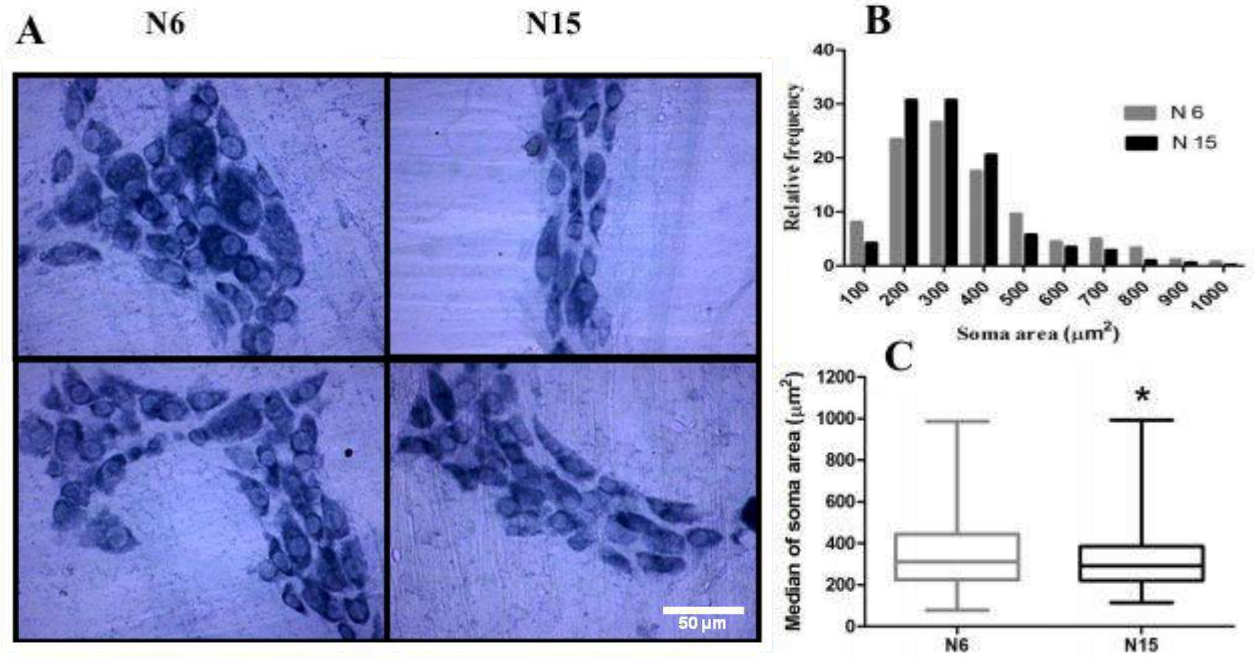


Figure 5 Myenteric plexus of undernourished animals contain smaller neurons. Representative photomicrographs of immunostaining of myenteric neurons immunoreactives for HuC/HuD (A). Histogram showing the distribuition of soma areas (B). Median of soma area between nourished (N6) and undernourished (N15) groups (C). n = 5 per group. Mann Whitney test, p = 0,026. Data are presented as median ± interquartiles.

Figure 6

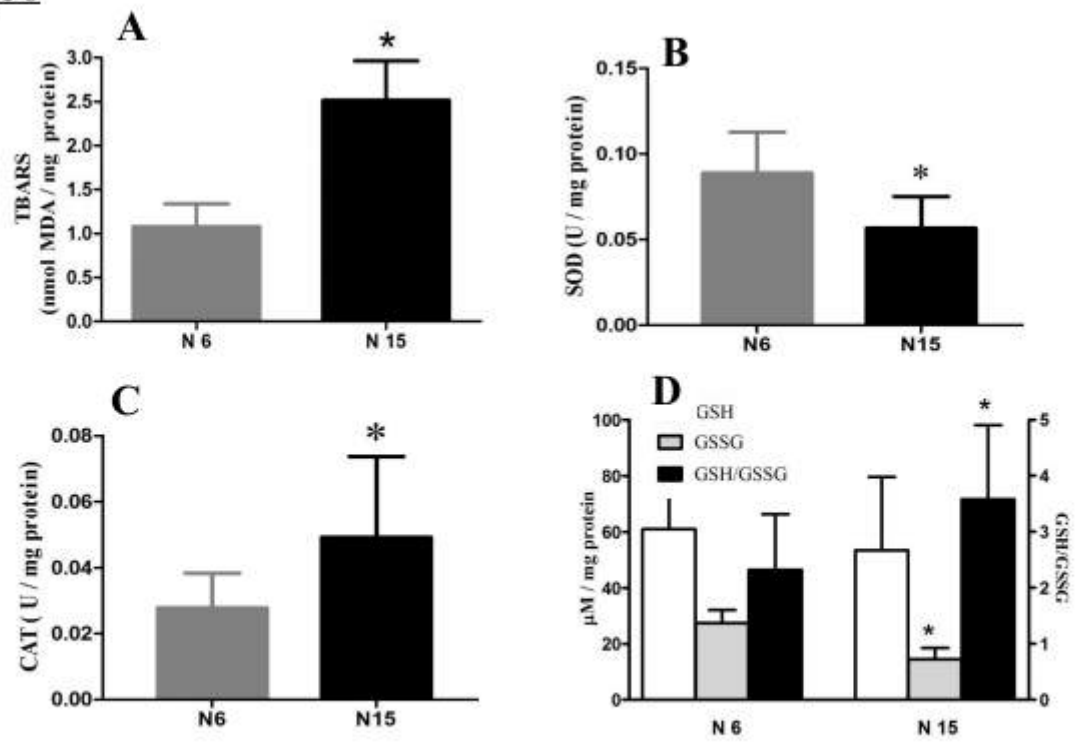


Figure 6. Oxidative status of distal colon is modified by undernutrition during lactation. Levels of lipoperoxidation (TBARS) (A), total super oxide dismutase (tSOD) (B) and catalase (CAT) (C) acitivities; reduced (GSH) and oxidized (GSSG) gluthathione (D) between control (N6) and undernourished rats (N15). n=7 per group. Unpaired t test.* p < 0,001. Data are presented as mean ± SD

Figure 7

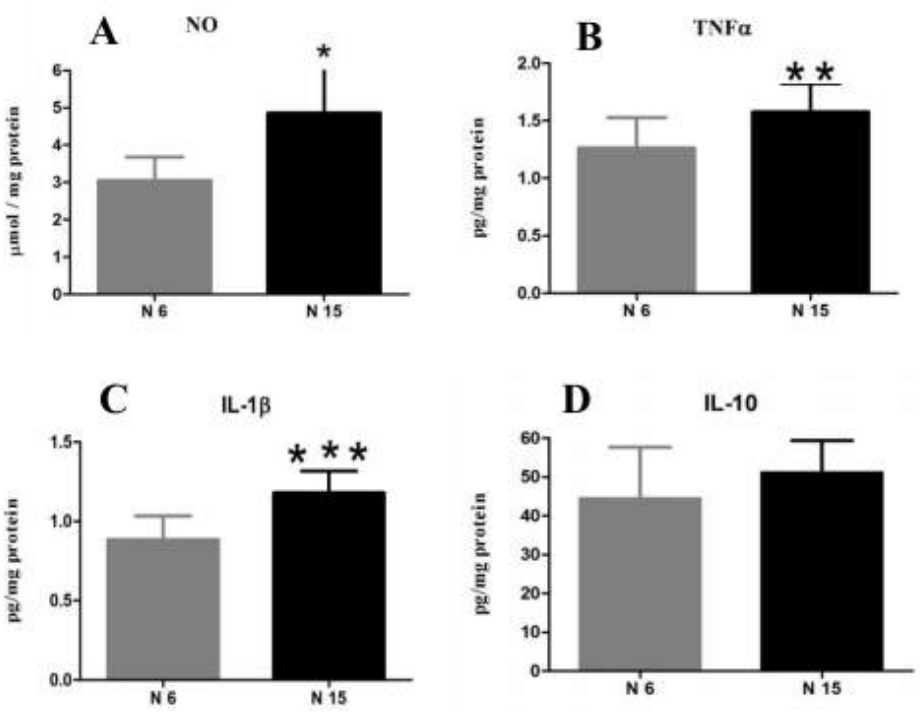


Figure 7. Moderate inflammatory status is induced in the colon of undernourished animals. Levels of nitric oxide (NO) (A), tumor necrosis factor α (TNFα) (B), Interleukin 1β (IL-1 β) (C) and interleukin 10 (IL-10) (D) between control (N6) and undernourished rats (N15). n=7 per group. Unpaired t test.* p = 0,042, ** p = 0,03, ***p = 0,002. Data are presented as mean ± SD

Figure 8

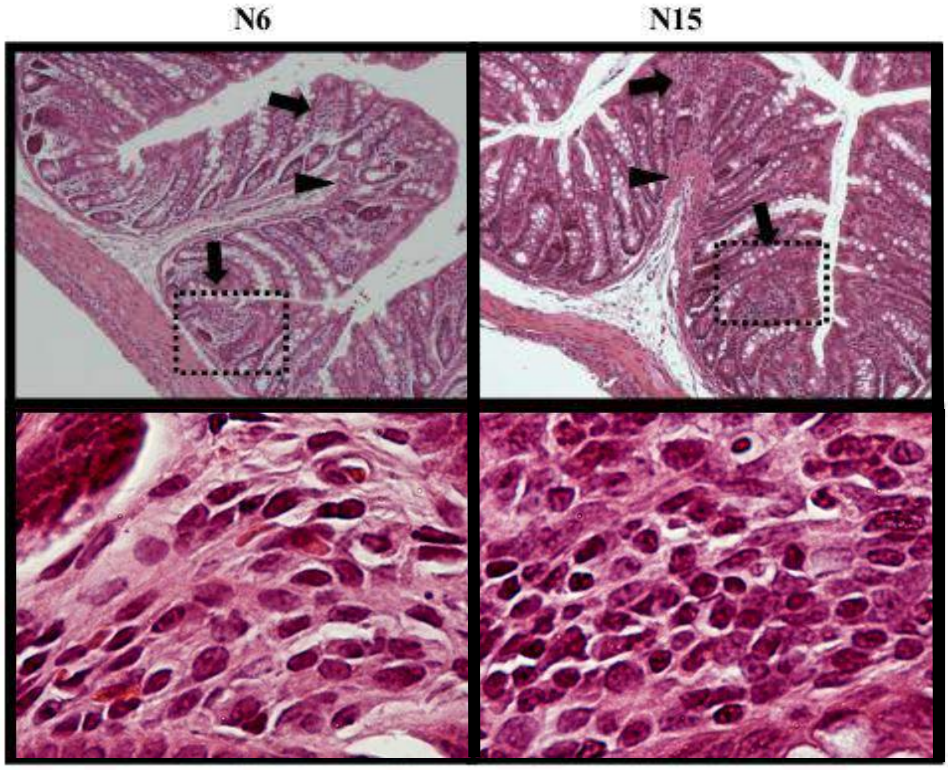
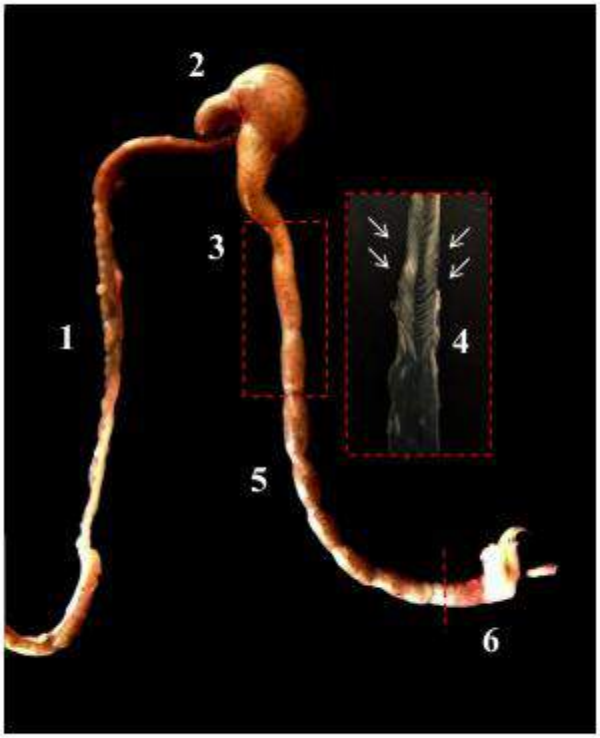


Figure 8. Structural evidence of gut inflammation. Representative images of distal colon from rats nourished (N6) and undernourished (N15) stained with hematoxylin and eosin. Arrows indicate the presence of lymphocytes in both groups, but especially a higher density in the experimental group (high magnification) as well as a larger muscle thickening of the submucosal layer (arrowhead)

Supplementary figure



1- Ileum

2- Cecum

3- Proximal colon

4- Limit between proximal and distal colon.

Mucosal oblique folds (arrows)

5- Distal colon

6- Rectum

Representative photo showing part of the small intestine and the large intestine complete, of an animal at 25 days, evidenced that limits used for analysis of the distal colon.

6. CONSIDERAÇÕES FINAIS

Os achados reforçam a importância de uma condição nutricional adequada no estágio inicial da vida, repercutindo para o desenvolvimento saudável do organismo de uma maneira geral, bem como do sistema nervoso entérico e do cólon dos animais, nas primeiras semanas pós nascimento.

Considerando a importância dos nutrientes do leite materno para um desenvolvimento adequado do sistema nervoso entérico, o presente estudo testou a hipótese de que a redução da alimentação durante o aleitamento é capaz de prejudicar a estrutura e função dos neurônios entéricos e células gliais, a indução de estresse oxidativo e estado inflamatório no cólon de ratos recém-desmamados.

Os resultados apoiam parcialmente a nossa hipótese, demonstrando que a lipoperoxidação, a produção de óxido nítrico e marcadores pró-inflamatórias, tais como TNF-α e IL-1β foram aumentadas por este insulto nutricional. Desequilíbrio nas enzimas antioxidantes também foi detectado. Por outro lado, a IL-10 e GSH não foram modificada e surpreendentemente GSH / GSSG foi aumentada.

Tomados em conjunto, os dados sugerem potenciais mecanismos pelos quais as modificações na condição anti-oxidativa e anti-inflamatória do intestino grosso no início da vida podem induzir a vulnerabilidade do cólon distal a estímulos adversos no futuro. Estudos funcionais precisam ser feitos para comprovar, no entanto, é possível que estes mecanismos possam aumentar o risco de doenças do intestino a longo prazo.

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APÊNDICES

APÊNDICE A - Artigo publicado na “Biochimica et Biophysica Acta”: Omega-3 deficiency and neurodegeneration in the substantia nigra: Involvement of increased nitric oxide production and reduced BDNF expression

Biochimica et Biophysica Acta 1840 (2014) 1902–1912

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Omega-3 deficiency and neurodegeneration in the substantia nigra: Involvement of increased nitric oxide production and reduced BDNF expression

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ARTICLE INFO

Article history:
Received 28 June 2013
Received in revised form 1 December 2013
Accepted 16 December 2013
Available online 20 December 2013

Keywords:
Brain-derived neurotrophic factor
Lipoperoxidation
Docosahexaenoic acid
Dopamine
Oxidative stress
Striatum

ABSTRACT

Background: Our previous study demonstrated that essential fatty acid (EFA) dietary restriction over two generations induced midbrain dopaminergic cell loss and oxidative stress in the substantia nigra (SN) but not in the striatum of young rats. In the present study we hypothesized that omega-3 deficiency until adulthood would reduce striatum's resilience, increase nitric oxide (NO) levels and the number of BDNF-expressing neurons, both potential mechanisms involved in SN neurodegeneration.
Methods: Second generation rats were raised from gestation on control or EFA-restricted diets until young or adulthood. Lipoperoxidation, NO content, total superoxide dismutase (t-SOD) and catalase enzymatic activities were assessed in the SN and striatum. The number of tyrosine hydroxylase (TH)- and BDNF-expressing neurons was analyzed in the SN.
Results: Increased NO levels were observed in the striatum of both young and adult EFA-deficient animals but not in the SN, despite a similar omega-3 depletion (~65%) in these regions. Increased lipoperoxidation and decreased catalase activity were found in both regions, while lower tSOD activity was observed only in the striatum. Fewer TH- (~40%) and BDNF-positive cells (~20%) were detected at the SN compared to the control.
Conclusion: The present findings demonstrate a differential effect of omega-3 deficiency on NO production in the rat's nigrostriatal system. Prolonging omega-3 depletion until adulthood impaired striatum's anti-oxidant resources and BDNF distribution in the SN, worsening dopaminergic cell degeneration.
General significance: Omega-3 deficiency can reduce the nigrostriatal system's ability to maintain homeostasis under oxidative conditions, which may enhance the risk of Parkinson's disease.

1. Introduction

The vulnerability of nigrostriatal dopaminergic neurons to lesions has been a matter of discussion and investigation in early and recent studies, especially due to the relevance of these cells in the etiology of Parkinson's disease [1,2]. It is well established that under physiological conditions, the substantia nigra (SN) has unique biochemical features which leads to a higher vulnerability to oxidative stress (OS) when compared to other brain regions [3]. Moreover, inflammation, excitotoxicity and metabolic aspects specific to dopaminergic cells have also been cited as potential cellular mechanisms underlying degeneration of these cells under certain pathological conditions [2].

The SN exhibits a high concentration of microglia [4] and the overactivation of these cells can result in the release of cytokines and free radicals such as superoxide radicals and nitric oxide (NO) [5,6]. These bioactive molecules released from microglia have been thought to contribute to SN dopaminergic cell death induced by mitochondrial dysfunction [7] or by lipopolysaccharide-induced inflammation [8].

An intrinsic neuronal population containing nitric oxide synthase (NOS) and nitrergic afferent neurons from the pedunculopontine tegmental nucleus (PPTg) is also present in the SN [9]. The potential

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http://dx.doi.org/10.1016/j.bbagen.2013.12.023

involvement of neuronal NOS (nNOS) activity in nigral cell degeneration has been discussed not only due to the importance of NO as a physiological modulator of cortico-striatal glutamatergic activation but also because its synthesis in the striatum can be modulated by dopamine receptor subtypes D1 and D2 [9–11].

A growing body of evidence indicates that dopamine mesostriatal and mesolimbic systems are also particularly vulnerable to reduced levels of long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) from the omega-3 family. Modifications on the dopamine metabolism in the frontal cortex, hippocampus, amygdala, nucleus accumbens [12–14] and dopaminergic cell loss in the SN pars compacta [15,16] have been described as a consequence of this nutritional deficiency. Conversely, in experimental models of Parkinson's disease, dietary DHA supplementation partially restored dopaminergic neurotransmission after 6-hydroxidopamine (6-OHDA)—or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced striatal lesions [17,18]. Recent studies have also linked DHA and the expression of neurotrophins involved in the development and survival of midbrain neurons. For example, dietary DHA supplementation was able to increase glial cell-derived neurotrophic factor and neurturin in the SN, reducing dopaminergic cell death induced by MPTP [19]; as well as increased brain-derived neurotrophic factor (BDNF) mRNA expression in mouse striatum [20]. The influence of DHA on BDNF levels and/or activity of its receptor TrkB has also been reported in the hippocampus, cerebral cortex [21] and spinal cord [22].

Among other factors with SN activity, only BDNF is both a potent dopaminergic and GABAergic neurotrophin [23,24] and normally expressed in high levels in the adult nigrostriatal system [25,26] exerting also neuroprotection in the aging brain [27,28]. Although mRNA for BDNF is present in the striatum, experimental studies using colchicine treatment demonstrated that BDNF is produced in the SN and is anterogradely transported to the striatum [29]. Thus, it has been discussed that BDNF may be acting as an autocrine/paracrine regulator, modulating striatal dopaminergic innervation and sprouting as well as neuron survival in the SN [23,24]. Consistent with such neuroprotective roles, intrathecal infusion of BDNF reduced the loss of dopamine neurons and the severity of Parkinson's disease in MPTP treated monkeys [30]. Evidence in humans has demonstrated that BDNF mRNA expression in the SN [31] and BDNF protein levels in the caudate, putamen and SN are reduced in patients with Parkinson's disease [32]. Furthermore, chronic deprivation of TrkB signaling leads to selective late onset of nigrostriatal dopaminergic degeneration [33].

Previous studies from our laboratory, adopting a two-generation model of essential fatty acid (EFA) dietary restriction, demonstrated that this type of experimental model was able to induce similar levels of DHA depletion in the SN, striatum and cerebral cortex of young animals (30–42 days). In the SN of these animals, 50% DHA depletion significantly reduced tyrosine hydroxylase (TH) protein levels, the size of dopaminergic cells and the total number of these neurons in the rostro-dorso-medial (SNrm) but not in the caudo-ventro-lateral region of SN (SNcv) [16]. When analyzing potential mechanisms involved in these deleterious effects, it was demonstrated that signs of neurodegeneration in dopaminergic and non-dopaminergic neurons, increased levels of lipoperoxidation (LP) and reduced enzymatic anti-oxidant resource were detected in the SN but not in the striatum [34]. In the present study, using the same experimental model, we decided to investigate whether increased nitric oxide levels could contribute to SN oxidative stress induced by EFA dietary restriction in young animals and extended our analysis to adult animals. Considering that adequate levels of omega-3 fatty acids during gestation and throughout maturation of the central nervous system are crucial for building neural resilience during adulthood, we also hypothesized that increasing DHA depletion in adult animals could affect dopaminergic neurons in the SNcv and the redox balance in the striatum. Furthermore, taking into account the importance of BDNF paracrine action on SN dopaminergic and non-dopaminergic cells, we also analyzed how the number of SN's BDNF-expressing cells could be affected by this type of dietary restriction.

2. Materials and methods

2.1. Animals, diets and tissue processing

All procedures were approved by the Ethics Committee for Animal Research of Federal University of Pernambuco (protocol # 009428/200633), which complies with the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA). Adult female *Wistar* rats weighing 200–250 g were fed from mating throughout pregnancy and lactation on control or experimental diets, each containing approximately 400 kcal/100 g and differing only in the lipid source. The diets were prepared according to Soares et al. [35] and met all current nutrient standards for rat pregnancy and growth as shown in Table 1.

As previously described [34] and herein shown in Supplementary Table 1, the control diet contained 50 g/kg of soybean oil with adequate amounts of saturated (26.01%), monounsaturated (12.32%), α-linolenic (6.04%) and linoleic (55.36%) acids. The experimental diet contained 50 g/kg of coconut oil (from Babaçu, *Orbignya martiana*) with reduced levels of linoleic (8.10%) and α-linolenic (0.49%) acids and higher levels of saturated (62.29%) and monounsaturated (23.73%) fatty acids. This diet is commonly used to induce omega-3 and omega-6 deficiency in several organs and systems [36–39]. Nevertheless, despite its reduced level of linoleic acid, Ling et al. in 2010 and our previous studies [16,34,40] demonstrated that arachidonic acid levels are not affected and only DHA is significantly reduced in the brain. For this reason, we used this diet as an experimental model of omega-3 deficiency for the nervous system.

Rat male offspring ($n = 112$) were the object of the present study. At parturition, litter size, total litter weight and mean birth weight of the pups were recorded. Litters containing at most 13 pups were used and reduced to 6 pups each, on postnatal day 1, by keeping the median weighted animals. Dams and pups were distributed into two main groups according to the nutritional condition: control (C) and deficient (D) rats. After weaning, at postnatal day 21, pups were separated and fed ad libitum the same diet as their respective mothers. First generation males and females were allowed to mate, to provide the second generation young animals (30–42 days old, CY and DY groups) and adult animals (90–110 days old, CA and DA groups). A total of 25 young and 87 adult animals were used. In each group, animals were

Table 1
Diet composition (grams/100 g diet).

Ingredients	Control diet	Experimental diet
Casein	20.7	20.7
Cellulose	1.8	1.8
Corn starch	46.8	46.8
Sucrose	21.0	21.0
Soybean oil	5.0	–
Coconut oil	–	5.0
Vitamin mix[a]	0.9	0.9
Mineral mix[b]	3.7	3.7
D,L-Cystine	0.1	0.1
Butyl hydroxytoluene	0.001	0.001
kcal/100 g	399.1	400.5

[a] Vitamin mixture (Rhoster Ind. Com. LTDA. SP. Brazil) containing (m%): folic acid (20); niacin (300); biotin (2); calcium pantothenate 160; pyridoxine (70); riboflavin (60); thiamine chloride (60); vitamin B_{12} (0.25); vitamin K_1 (7.5). Additionally containing (UI%): vitamin A 40.000; vitamin D_3 10.000; and vitamin E (750).

[b] Mineral mixture (Rhoster Ind. Com. LTDA. SP. Brazil) containing (m%): $CaHP0_4$ (38); K_2HP0_4 (24); $CaCO_3$ (18.1); NaF (0.1); NaCl (7.0); MgO (2.0); $MgS0_4$ $7H_2O$ (9.0); $FeS0_4$ $7H_2O$ (0.7); $ZnS0_4$ H_2O (0.5); $MnSO + H_2O$ (0.5); $CuS0_4$ $5H_2O$ (0.1); Al_2 $(S0_4)_3K_2S0_4$ $24H_2O$ (0.02); Na_2SeO_3 $5H_2O$ (0.001); and KCl (0.008).

randomly sampled from different litters, housed three per cage in a room maintained at 22 ± 2 °C with 67% relative air humidity and kept on a 12 h light/dark cycle (lights on at 6:00 h). Each experimental day, three or six animals per group, from three different litters, were anesthetized with isofluorane and then decapitated. The preanesthesia with volatile agents to prevent pain and reflexes is recognized by animal welfare regulatory agencies (e.g. CONCEA, Brazil; UK Animals Scientific Procedure, 1986) and many biochemical and electrophysiological laboratories use this procedure routinely (e.g. Randall et al; [41]). We adopted this procedure because it greatly prevents sudden rise in plasma catecholamines and cortisol due to environmental clues of eminent death. It also avoids unfortunate accidents like the animal moving its head just before being guillotined. Davis [42] investigated the effect of isoflurane anesthesia on metabolites in the rat prior to decapitation and there were no significant effects of this anesthesia with regard to plasma and liver carnitine, plasma beta-hydroxybutyrate and free fatty acids, or liver free fatty acids, triglycerides, free Coenzime A, and acetyl coenzyme A. Additionally, we previously have compared experiments conducted with or without the use of volatile anesthetic, and results were not affected (data not shown).

After decapitation, the regions containing the SN or striatum were rapidly dissected in a 0.9% (w/v) NaCl solution at 4 °C. After weighing, the pooled tissue was homogenized in a 0.9% (w/v) NaCl solution (1:10) at 4 °C and centrifuged for 10 min at 1000 *g* at 4 °C for an analysis of LP via determination of thiobarbituric acid-reactive substance (TBAR) levels and for 10 min at 10,000 *g* at 4 °C in order to assess either the total (Cu–Zn and Mn) superoxide dismutase (t-SOD) and catalase enzymatic activities, as well as nitric oxide levels via nitrite measurement. An aliquot of supernatant was analyzed for total protein content using a bicinchoninic acid protein kit (Sigma-Aldrich, St. Louis, MO).

2.2. Lipid peroxidation (LP) quantification

LP was measured by estimating malondialdehyde (MDA) using a thiobarbituric acid (TBA) reaction (TBAR method) according to Ohkawa et al., [43]. In the TBA test reaction, MDA or MDA-like substances and TBA react to produce a pink pigment with maximum absorption at 532 nm. The reaction was developed by the sequential addition of 0.2 mL of 8.1% sodium duodecil sulfate, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% TBA solutions to triplicates of supernatants in a boiling water-bath for 30 min. After tap water cooling, 1.5 mL of n-buthanol/pyridine (15:1 v/v) was added to the samples, centrifuged at 2500 *g* for 10 min and the organic phase was read at 532 nm using a plate reader. The results were expressed as nmol/mg of protein using a standard curve generated with different concentrations of 1,1,3,3-tetramethoxypropane solution. Control SN and striatum samples were also incubated in a 30 μM sodium nitroprusside (SNP) solution for 45 min before the assay and used as positive controls for lipid peroxidation.

2.3. Total superoxide dismutase (t-SOD) activity

Assessment of t-SOD enzymatic activity was performed according to Misra and Fridovich [44] at 25 °C. Triplicates of SN or striatum supernatants (100 μL) had been previously incubated in a water bath at 37 °C and then added to an 880 μL solution of 0.05% sodium carbonate with a pH of 10.2 in 0.1 mM EDTA. The reaction was developed by adding 20 μL of 30 mM epinephrine in 0.05% acetic acid. The absorbance was measured at 480 nm for 4 min. One unit of t-SOD was defined as the enzyme amount responsible for 50% of the inhibition of epinephrine oxidation. Tissue t-SOD enzymatic activity was expressed as units (U)/mg of protein. Positive controls were obtained by incubating control homogenate samples of SN and striatum in a 30 μM SNP solution for 45 min before the enzymatic assay.

2.4. Catalase (CAT) activity

CAT activity was measured according to Aebi [45]. The rate constant k of H_2O_2 decomposition under our experimental conditions of temperature ~20 °C and pH 7.0 was determined to be 4.6×10^7, by measuring the absorbance changes per minute for 4 min. The enzymatic activity was expressed as the H_2O_2 consumed in nM/min/mg protein. Positive controls for CAT activity were obtained by incubation of SN and striatum homogenates of the control group in increasing concentrations of H_2O_2 (3.156 to 100 μM) for 30 min at 37 °C before the enzymatic assay, as previously described [34].

2.5. Estimation of nitrite concentration

Nitrite levels were estimated using the Griess reagent which served as an indicator of nitric oxide production as described by Green et al. [46]. Equal volumes (100 μL) of supernatant and reagent (1% sulfanilamide in 2.5% phosphoric acid and 0.1% N-(1-naphthyletilene diamine dihydrochloride in water)) were placed in 96-well plates and reacted for 10 min at room temperature (~20 °C). The absorbance of diazonium compound was measured at a wavelength of 540 nm. The results were expressed as μmol nitrite/mg of protein with reference to a standard curve built with sodium nitrite concentrations.

2.6. Tyrosine hydroxilase (TH) and BDNF immunohistochemistry

Rats (six animals per group) were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially first with saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4, 200 mL). Perfusion was always performed between 12:00 and 18:00 h, with a continuous infusion pump (Harvard equipment). After perfusion, the brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem (the olfactory bulb and cochleas were excluded). They were then postfixed for 2 h in the same fixative, rinsed in PB and weighed (wet weight). Subsequently, the brains were cryoprotected in sequential solutions of 10%, 20% and 30% sucrose in PB. Brain blocks were serially cut on a cryostate (Leica) into 50 μm-thick sections across the parasagittal plane of each hemibrain. All sections were collected serially in PB and arranged in six series. The Paxinos and Watson stereotaxic atlas [47] was used to delimit the cytoarchitectonic regions. The series of sections used for BDNF or TH immunohistochemistry were treated with a 0.1 M borate buffer (pH 9.0) or 0.01 M citrate buffer (pH 6.0) at 60 °C for 1 h. Thereafter, free-floating sections were rinsed in PB and incubated with a rabbit anti-BDNF polyclonal antibody (Santa Cruz, USA; 1:200) or a rabbit anti-TH polyclonal antibody (Chemicon, USA; 1:500) diluted in PB containing 0.3% Triton X-100 (PBX) and 1% normal goat serum for 24 h at 4 °C. Sections were then incubated for 1 h in secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs, USA) diluted 1:200 in PBX, and processed for immunoperoxidase staining using the avidin–biotin–peroxidase complex (Standard ABC kit, Vector Labs). Antibody binding was revealed with diaminobenzidine tetrahydrochloride 0.05% (DAB, Sigma). Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated glass slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. As for the control of the staining specificity, some sections were subjected to the immunohistochemical procedure omitting the primary antiserum.

Double-staining for BDNF and TH in brain sections of three CA and DA animals was performed in order to identify the distribution of double and non-double labeled cells in the SN. BDNF-positive cells were stained using the DAB reaction and then the TH-positive neurons were labeled with DyLight-conjugated 488-labeled anti-rabbit IgG (1:1000, Rockland) for 24 h. After incubation with the fluorophore-coupled antibody, the sections were washed three times in PB, mounted onto gelatin coated glass slides, dried at 50 °C for 30 min, cleared in xylene for 1 min

and mounted with Entellan (Merck). The analysis was carried out using an epifluorescence microscope (Leica, DMLB).

2.7. Stereological quantification and morphometric analysis of TH and BDNF positive cells in the substantia nigra

Total estimates of the number of TH and BDNF positive cells in the SN were obtained from five brains per group. Four and three parasagittal sections from a 1:6 series immunoreacted for TH or BDNF, respectively, were analyzed for each brain. BDNF cells were quantified along the whole extension of SN and TH positive cells from SNrm or SNcv were analyzed separately. Optical fractionator sampling was carried out using a Nikon Eclipse 80 microscope equipped with an advanced scientific instrumentation motorized stage input into a high resolution plasma monitor and linked to a MBF CX 9000 color digital video camera. Sampling used to count stained cells was implemented using Stereoinvestigator software (MicroBrightField Inc.; Williston, USA). Areas of interest containing TH and BDNF positive cells were outlined with reference to an atlas of the rat brain [47]. The stereology was performed at high magnification with a 100×/1.4 aperture oil immersion lens which allows clear visualization of the nucleus and precise definition of the cell walls, according to a protocol previously tested and published for parasagittal sections immunoreacted for TH in second generation young rats [16]. The coefficient of error (CE) expresses the accuracy of the cell number estimates and a value of CE ≤ 0.10 was deemed appropriate for the present study, because variance introduced by the estimation procedure contributes little to the observed group variance [48]. The experimental parameters adopted for the stereological analysis are shown in Supplementary Tables 2 and 3.

2.8. Soma size of dopaminergic cells

Cell body areas of TH-immunoreactive neurons in the SNrm or SNcv of adult animals were measured using the Neurolucida System for Neuroanatomical Analysis (MicroBrightField Inc.; Williston, USA). To delimit the outlines of cell somata, a systematic random sampling of cells was made using high magnification images (with 100×/1.3 aperture oil immersion lenses) whenever the cell nucleus could be clearly identified. These measurements were carried out on six animals per group, in the left side of the brain. Five parasagittal sections from lateral to medial levels of SN were analyzed per animal. In the SNrm or SNcv, a minimum number of 50 cells per region/animal were set to be analyzed. Thus, a total of ~300 cells were analyzed per group in the SNrm or SNcv.

2.9. Fatty acid determination in the striatum and midbrain

The fatty acid profiles of striatum and midbrain phospholipids were assessed in F2 groups at 95 days of age. Animals (n = 6/group) were decapitated and the regions containing the midbrain were rapidly dissected in an ice bath. The tissues were homogenized in a 50 mM Tris–HCl buffer (pH 7.4) with EGTA and centrifuged for 30 min at 28000 g at 4 °C. The pellets were immediately re-suspended in 50 mM Tris–HCl buffer (pH 7.4). The total phospholipids were extracted and transmethylated as previously described [34]. The fatty acid profile was analyzed using a Shimadzu GC apparatus equipped with a flame ionization detector and HP-inowax 20M capillary column (30 m × 0.32 mm × 0.3 μm). The column temperature was initially 40 °C for 1 min, then increased to 150 °C by 55 °C/min, and finally increased to 220 °C by 1.7 °C/min. The injector and detector temperatures were 200 and 220 °C, respectively. Hydrogen was used as the carrier gas at a flow rate of 1.0 mL/min; injection was in split-less mode and the injection volume was 1.0 μL of the sample isooctane extract. A standard fatty acid methyl ester mixture (SupelcoTM, 37 Component FAME mix, USA) was used to identify the fatty acid methyl esters by their retention time. Fatty acid data were expressed as percentage of total peak area. Differences between the groups were analyzed by Student's t test and considered significant at $p < 0.05$.

2.10. Statistical analysis

Biochemical data of TBAR levels, t-SOD and CAT enzymatic activity, and nitrite concentration were plotted using GraphPad Prism Software, version 5.0 for Windows (San Diego, CA, USA) and the statistical analysis was performed using ANOVA followed by Tukey as the post-hoc test or Student's t-test. The analysis of body, brain weight and the number of TH or BDNF positive cells was carried out using unpaired Student's t-test. The non-parametric Kruskal–Wallis ANOVA Ranks test was used to analyze the effects of diets and regions on the measures of neuronal soma size and the Dunn's test, $\alpha < 0.05$ was further utilized to determine post-hoc comparison among groups and regions. Unless stated otherwise, all values are expressed as mean ± standard deviation (SD).

3. Results

3.1. Body and brain weights

Body weights of young and adult animals were significantly smaller in young (DY) and adult (DA) deficient groups as compared to the respective controls. Brain weights of DY but not of DA animals, were significantly smaller, compared to control. Nevertheless, the brain weight/body weight ratio did not differ between the groups in each age (Table 2).

3.2. EFA dietary restriction over two generations induces similar DHA depletion in the striatum and substantia nigra of adult animals

Analysis of the brain fatty acid profiles of adult animals (Table 3) demonstrated that the DHA (22:6n−3) levels in phospholipids of the DA group was in average ~65% lower than that obtained in the respective controls. DHA deficiency was also confirmed by a significant increase in the docosapentaenoic fatty acid (DPA; 22:5n6) levels in both SN and in the striatum (2-tail t-test, $p < 0.001$). On the other hand, the values for arachidonic acid (AA; 20:4n−6) did not differ between control and experimental animals, neither in the SN nor in the striatum as was previously demonstrated in young animals [16,34]. Regarding saturated and monounsaturated fatty acids, the presence of coconut oil in the maternal diet significantly increased the levels of palmitic (16:0) acid (2-tail t-test, $p < 0.01$) in both SN and striatum phospholipids and oleic (18:1n9) acid in the SN compared to the control diet.

3.3. Lipid peroxidation, t-SOD and CAT enzyme activities are differentially modified in the SN and striatum of adult animals

As shown in Fig. 1A, evidence of increased lipid peroxidation, was detected in the SN and striatum of the DA group (0.82 ± 0.1 and 0.9 ± 0.18 nmol MDA/mg protein, respectively) in comparison with

Table 2
Body and brain weights.

Groups	Body weight (g)	Brain weight (g)	Brain weight/body weight ratio
CY	79.65 ± 14.87 (n = 33)	1.69 ± 0.06 (n = 08)	0.0210 ± 0.004 (n = 08)
DY	71.91 ± 10.09* (n = 40)	1.48 ± 0.12*** (n = 10)	0.0200 ± 0.010 (n = 08)
CA	385.46 ± 41.75 (n = 15)	2.05 ± 0.18 (n = 06)	0.0050 ± 0.004 (n = 08)
DA	338.28 ± 36.68** (n = 21)	1.91 ± 0.12 (n = 09)	0.0056 ± 0.005 (n = 08)

Values are expressed as Mean ± SD. Unpaired Student's t test.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3
Fatty acid composition (% of total) in the striatum and midbrain membrane phospholipids of adult animals raised on control or experimental diets.

Fatty acid	Striatum		Midbrain	
	Control diet	Experimental diet	Control diet	Experimental diet
C16	17.10 ± 1.04	22.70 ± 0.74*	15.28 ± 1.90	23.10 ± 0.27*
C16:1	0.73 ± 0.10	0.76 ± 0.12	0.64 ± 0.04	0.85 ± 0.03
C17	nd	nd	0.16 ± 0.00	0.20 ± 0.02
C18	25.74 ± 0.25	25.50 ± 0.51	24.88 ± 0.67	25.10 ± 0.37
C18:1n9	14.00 ± 0.34	15.18 ± 0.69	16.38 ± 0.25	15.10 ± 0.21*
C18:2n6t	nd	nd	0.74 ± 0.17	0.65 ± 0.22
C20:1	nd	nd	0.62 ± 0.10	0.60 ± 0.05
C20:4n6 (AA)	14.08 ± 0.27	13.10 ± 0.34	13.74 ± 0.83	13.90 ± 0.48
C23	3.75 ± 0.13	3.46 ± 0.18	3.98 ± 0.20	2.50 ± 0.19
C22:5n6 (DPA)	1.35 ± 0.06	11.30 ± 0.36**	1.45 ± 0.05	10.60 ± 0.46**
C22:6n3 (DHA)	23.25 ± 0.51	8.00 ± 0.41**	22.12 ± 0.21	7.50 ± 0.31**

Values are expressed as means ± SD. nd = not detected.
* $P < 0.01$.
** $P < 0.001$.

the respective control group (0.4 ± 0.03 and 0.35 ± 0.02 nmol MDA/mg protein, $p < 0.05$). Thus, the magnitude of lipid peroxidation induced by the experimental condition in the SN or striatum is, respectively, 32 and 25% less than that obtained by using 30 μM SNP (1.2 ± 0.143 nmol MDA/mg protein). A significant decrease in t-SOD enzyme activity was found in the striatum of the DA group (0.05 ± 0.1 U/mg protein) compared to the control group in the absence of pre-treatment with SNP (0.19 ± 0.2 U/mg protein, $p < 0.01$). No difference was detected between the groups for t-SOD activity in the SN (0.18 ± 0.05 and 0.14 ± 0.01 U/mg protein for DA and CA groups, respectively; Fig. 1B). On the other hand, the CAT activity was significantly reduced in the SN (2.38 ± 0.21 nmol/min/mg protein) and especially in the striatum (3.40 ± 0.60 nmol/min/mg protein) of the DA group compared to control groups (5.20 ± 1.11 and 9.58 ± 1.37 nmol/min/mg protein for SN and striatum respectively; $p < 0.001$; Fig. 1C).

3.4. EFA dietary restriction over two generations increases nitric oxide levels in the striatum but not in the SN of young and adult animals

Fig. 2 shows the results obtained with SN and striatum homogenates of young (Fig. 2A) and adult (Fig. 2B) animals. In the SN, no intergroup difference in the nitrite levels was observed either in young or in adult animals. However, in the striatum of both young (DY) and adult (DA) groups, nitrite levels were, respectively, 0.3-fold and 1.8-fold higher than those of the control group ($p < 0.001$). It is worth noting that in the striatum of the CA group, nitrite levels were estimated at about 3-fold higher than those in the SN of the same group.

3.5. EFA dietary restriction over two generations until adulthood reduces the number of TH positive neurons of both SNrm and SNcv

Panel A in Fig. 3 shows low magnification images of representative TH-immunoreactive parasagittal sections through mediolateral extent (lateral ~1.4 to ~2.8 mm according to the atlas of Paxinos and Watson [47]) of SNrm and SNcv in animals of CA and DA groups. As can be observed in panel A and reinforced in higher magnification images of panel B, fewer TH positive cells can be seen in both SNrm (Fig. 3Bc) and SNcv (Fig. 3Bd) of the DA animals, when compared to control ones. Comparative stereological analysis between the groups (Table 4) demonstrated that, on average, the number of TH-immunoreactive cells in rats fed the deficient diet was about 36% ($p < 0.001$) and 44% ($p < 0.05$) lower in the SNrm and SNcv, respectively, than in those fed the control diet. Combining the data of these two regions, a total of 21614.8 ± 3189.75 and 13320.8 ± 1807.15 dopaminergic neurons were estimated in the left SN of control and respective DA groups ($p < 0.001$).

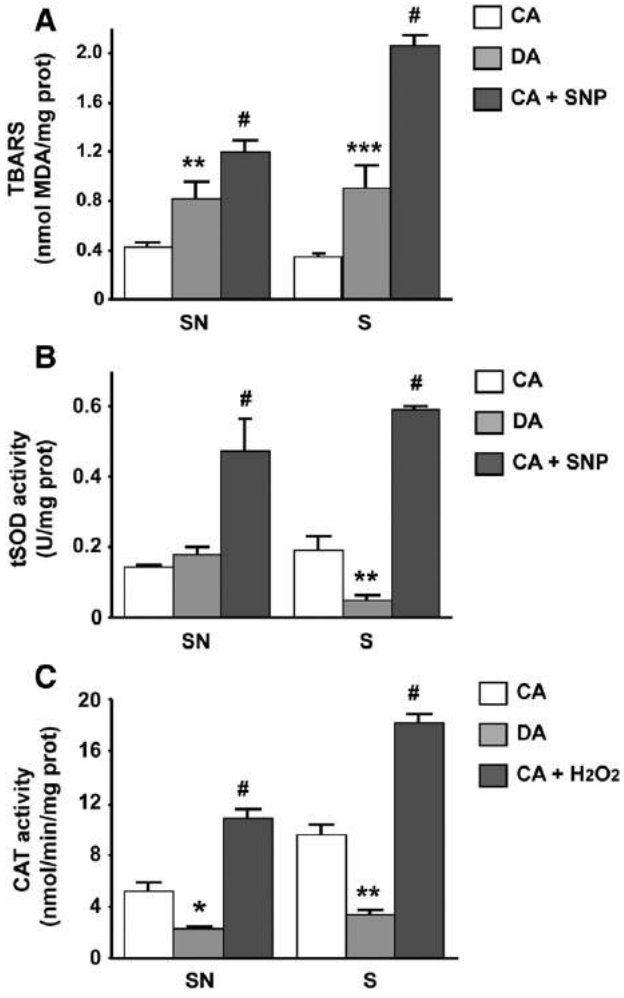

Fig. 1. Thiobarbituric acid-reactant substance (TBAR) levels (A), total superoxide dismutase (t-SOD) activities (B) and catalase (CAT) activities (C) in the substantia nigra (SN) and striatum (S) from adult rats fed EFA-restricted diet over two generations (DA) and respective controls (CA; $n = 12$ animals per group). *$P < 0.05$; **$P < 0.001$ compared to control group. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control in all the experiments. #$P < 0.0001$ compared to control or deficient group.

3.6. EFA dietary restriction over two generations reduces SN dopaminergic cell soma size in adult animals

The long term dietary treatment with the deficient diet until adulthood resulted in lower dopaminergic cell body size both in the SNrm (median = 244.031 μm^2 versus 203.308 μm^2 in the control) and the SNcv (median = 289.616 μm^2 versus 268.169 μm^2 in the control) according to Kruskal–Wallis ANOVA Ranks test, followed by the Dunn's test, $p < 0.001$ for both regions.

3.7. DHA deficiency reduces the number of BDNF expressing cells in the SN of adult animals

Fig. 4Aa and d illustrates low magnification images of representative brain parasagittal sections throughout the SN of CA and DA animals. As can be seen, BDNF-immunoreactivity is widely distributed throughout the entire extension of this nucleus. Fig. 4Ab, c, e and f shows higher magnification images of BDNF positive cells located in the SNrm (b and e) and SNcv (c and f) of both groups. Note that BDNF is present in a heterogeneous SN cell population, with respect to soma size and shape. The results of stereological analysis of BDNF expressing cells performed with 4 animals per group are shown in Table 5. This analysis demonstrated that the total number of SN BDNF-immunoreactive cells

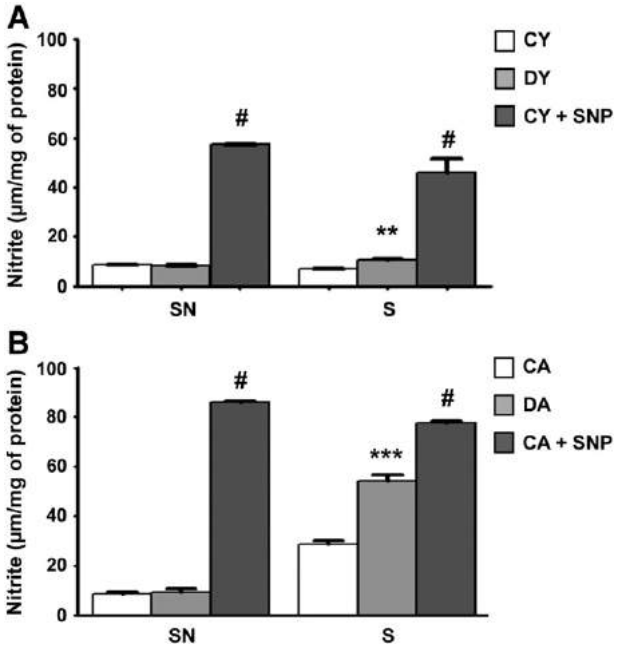

Fig. 2. Nitrite concentration as an indicator of nitric oxide production in the substantia nigra (SN) and striatum (S) from young (A) and adult (B) rats fed EFA- restricted diet over two generations (DA) and respective controls (CA; $n = 12$ animals per group). *P < 0.05; **P < 0.001 compared to control group. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control in all the experiments. #P < 0.0001 compared to control or deficient group. Nitrite concentration was determined by the Griess reagent.

in rats fed the deficient diet was ~20% lower than in those fed the control diet ($p < 0.05$). It should be noted that the Scheaffer coefficient of error (CE) was low and suitable in both groups analyzed. Nevertheless, as in the control group the coefficient of variation was smaller than CE, the biological coefficient of variation in this group became negative.

3.8. Double staining against TH and BDNF immunoreactive neurons in the SN

Double staining for BDNF (reacted with DAB) and TH (visualized with DyLight-conjugated 488-IgG) in representative brain sections of control and DA animals is shown in Fig. 5. As can be seen, single (Fig. 5A and B, B′) or double labeled cells (Fig. 5C and C′; D and D′; E and E′) were detected in the SN of both groups. Thus, remaining TH-positive cells in the SN of DHA deficient animals comprise a heterogeneous cell population regarding BDNF expression.

4. Discussion

The present study investigated whether the dietary restriction of essential fatty acids over two generations could reduce the number of BDNF positive cells and increase the nitric oxide levels in the SN as potential mechanisms involved in the neurodegeneration and lipoperoxidation previously demonstrated [34]. A multigenerational model of EFA restriction was used to induce an increasing and non-compensated DHA deficiency [49,50]. It was hypothesized that this long-term dietary treatment lasting until adulthood could reduce the striatum's resilience observed in young animals, affecting its redox balance. The results partially support our hypothesis, demonstrating that NO production and t-SOD activity in the SN and striatum were

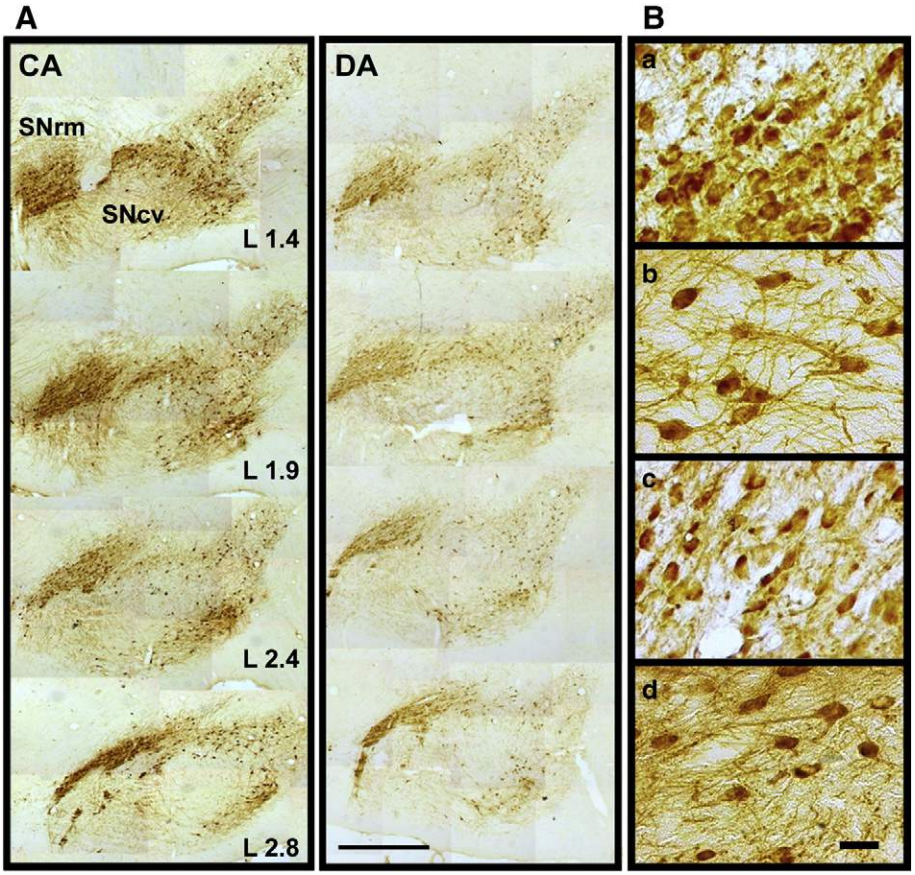

Fig. 3. (A) Representative low magnification images of TH-immunoreactive parasagittal sections though mediolateral extent of substantia nigra (SN) from adult rats fed control (CA) or EFA-restricted diet (DA) for two generations (scale bar = 500 µm; magnification power = 40×). (B) Higher magnification pictures show fewer dopaminergic cells in the substantia nigra rostro-dorso-medial (SNrm; Bc) and caudo-ventro-lateral (SNcv; Bd) in the DA group, compared to respective control (scale bar = 30 µm; magnification power = 200×).

Table 4
Estimated individual unilateral planimetric volumes of SNcv and SNrm and correspondent unilateral number of their respective dopaminergic cells.

Subjects	SNcv					SNrm					SNcv + SNrm
	Thickness	Volume (mm^3)	CE	Total cells	CE	Thickness	Volume (mm^3)	CE	Total cells	CE	Total cells
CF2 group											
C1	23.7	1.31	0.09	9550	0.083	22.8	0.4	0.11	17026	0.080	26576
C2	11.3	1.32	0.09	7500	0.085	25.1	0.3	0.11	14862	0.085	22362
C3	19.0	1.30	0.08	6914	0.087	21.3	0.3	0.09	13235	0.080	20149
C4	17.2	1.34	0.09	6320	0.085	20.2	0.3	0.09	14653	0.075	20973
C5	15.7	1.30	0.10	6240	0.084	18.0	0.3	0.10	11774	0.077	18014
Mean	17.38		0.09	7305	0.085	21.48	0.32	0.10	14310	0.0794	21614.8
SD	4.54		0.007	1354	0.001	2.67	0.044	0.001	1961	0.0038	3189.75
CV^2				0.034					0.018783863		
CE^2				0.0072					0.00630436		
CE^2/CV^2				0.212					0.335626383		
CVB^2				0.0268					0.012479503		
CVB^2 (%CV^2)				78.82%					66.44%		
EF2 group											
D1	14.1	1.4	0.10	4437	0.071	18.3	0.3	0.09	9160	0.075	13597
D2	13.1	1.3	0.14	3319	0.095	18.9	0.3	0.10	9142	0.072	12461
D3	15.5	1.2	0.10	3914	0.100	18.0	0.2	0.11	6804	0.087	10718
D4	14.6	1.3	0.10	4713	0.100	18.9	0.2	0.09	10650	0.068	15363
D5	14.1	1.6	0.09	4092	0.080	18.6	0.2	0.14	10373	0.078	14465
Mean	11.6	1.36	0.106	4095[#]	0.089	18.54	0.24	0.106	9225.8[#]	0.076	13320.8[#]
SD	1.9	0.151	0.01949	0.532	0.013	0.3911	0.05477	0.02073	1518.29	0.071	1807.15
CV^2				0.130014163					0.027083288		
CE^2				0.00795664					0.005776		
CE^2/CV^2				0.061198255					0.213268049		
CVB^2				0.122057523					0.021307288		
CVB^2 (%CV^2)				93.88%					78.67%		

CE, Scheaffer coefficient of error; CV, coefficient of variation; CVB, biological coefficient of variation; $CVB^2 = CV^2 - CE^2$; SD, standard deviation.
[#] $P < 0.05$ versus control.

differentially affected by this type of nutritional treatment. In addition, the reduced number of BDNF positive neurons in the SN of omega-3 deficient animals reinforces the involvement of this neurotrophin in DHA-induced neuroprotection.

As has been previously reported for young animals [34,51], the long term treatment with the EFA-restricted diet containing coconut oil was able to induce systemic effects on the rat somatic growth as shown by the reduced body weight detected in the adult animals. This data is consistent with the effect of diets containing coconut oils as the only source of lipids in reducing body weight gain. Such reduction seems to be independent of essential fatty acid deficiency [38]. On the other hand, no intergroup difference was observed in the brain weight of adult animals compared to that observed in young animals [16] which is in accordance with, previous studies adopting a dietary deficiency specific for α-linolenic fatty acid for two or three generations [52].

4.1. EFA dietary restriction over two generations until adulthood reduces striatum's resilience increasing nitric oxide and lipid peroxidation levels

The increasing DHA depletion in both SN and striatum of adult deficient groups (~65% reduction relative to control) was able to reduce the resilience of the striatum to oxidative insult, previously observed in young animals with ~50% DHA depletion [34]. Moreover, increased lipid peroxidation levels in both SN and striatum (~2 fold, compared to respective controls) also affected dopaminergic neurons located in the SNcv. Nevertheless, it should be noted that similar levels of DHA depletion in both SN and striatum induced distinct mechanisms underlying the oxidative stress herein described, especially those involving NO production.

Nitrergic terminals have been reported to make synaptic contacts with both SN dopaminergic neurons and their terminal areas such as the striatum [11]. An interesting piece of information obtained in the present study was that, in contrast to our initial hypothesis, increased midbrain DHA deficiency did not modify NO levels in the SN, neither at the young nor the adult stage, suggesting that modifications in the synthesis or release of this bioactive substance into this nucleus were not involved in the loss of dopaminergic cells. Studies on protective or deleterious effects of NO on neuronal survival have been widely debated in the literature [10,11,53]. While in physiological concentrations NO is able to prevent apoptotic events induced by hypoxia [54], in some neurodegenerative conditions, such as Parkinson's disease, a high concentration of NO leads to dopaminergic cell death [7,44]. An increased number of nNOS expressing neurons were observed in the SN after application of a non-excitotoxic neurotoxin in the PPTg nucleus, responsible for sending cholinergic, nitrergic and glutamatergic afferent neurons to the SN [9]. However, under such conditions, an increase in nNOS expression in the SN was not involved in cell degeneration in this nucleus [9]. A modulatory action of DHA on NO production has been discussed, indicating that the dietary supplementation of this fatty acid can reduce the activity of NOS in the striatum [55]. Our present findings, showing that DHA depletion did not change NO contents in the SN under conditions of oxidative stress, deserve further studies, especially to investigate whether this dietary treatment could reduce the number of nitrergic neurons and glial cell reactivity in this nucleus. This latter point is currently under investigation.

On the other hand, in the striatum, higher NO levels in young and adult deficient animals occurred with different magnitude and conditions of homeostatic response. While a 0.3-fold rise in NO content was detected in DY animals, where the t-SOD enzyme was reactive and increased LP was not observed [34], a greater elevation of NO production (~1.8 fold) was found in the DA group in a context with reduced t-SOD and CAT activities, and the LP twice as high as in the control condition. In the striatum, nitrergic interneurons are involved in corticoestriatal glutamatergic excitability and NO synthesis can be modulated by D1 and D2 dopamine receptor subtypes [11]. An increase in the nNOS cell density or NADPH-diaphorase activity in the striatum after chronic nigrostriatal deafferentation has been reported [56,57]. Moreover, under conditions of oxidative stress induced by 6-OHDA into the striatum, pretreatment with a NO donor worsened the dopamine cell degeneration in the SN [58]. A recent study has indicated reduced NO production in reactive microglia upon DHA supplementation as one potential mechanism involved in its anti-inflammatory action [59].

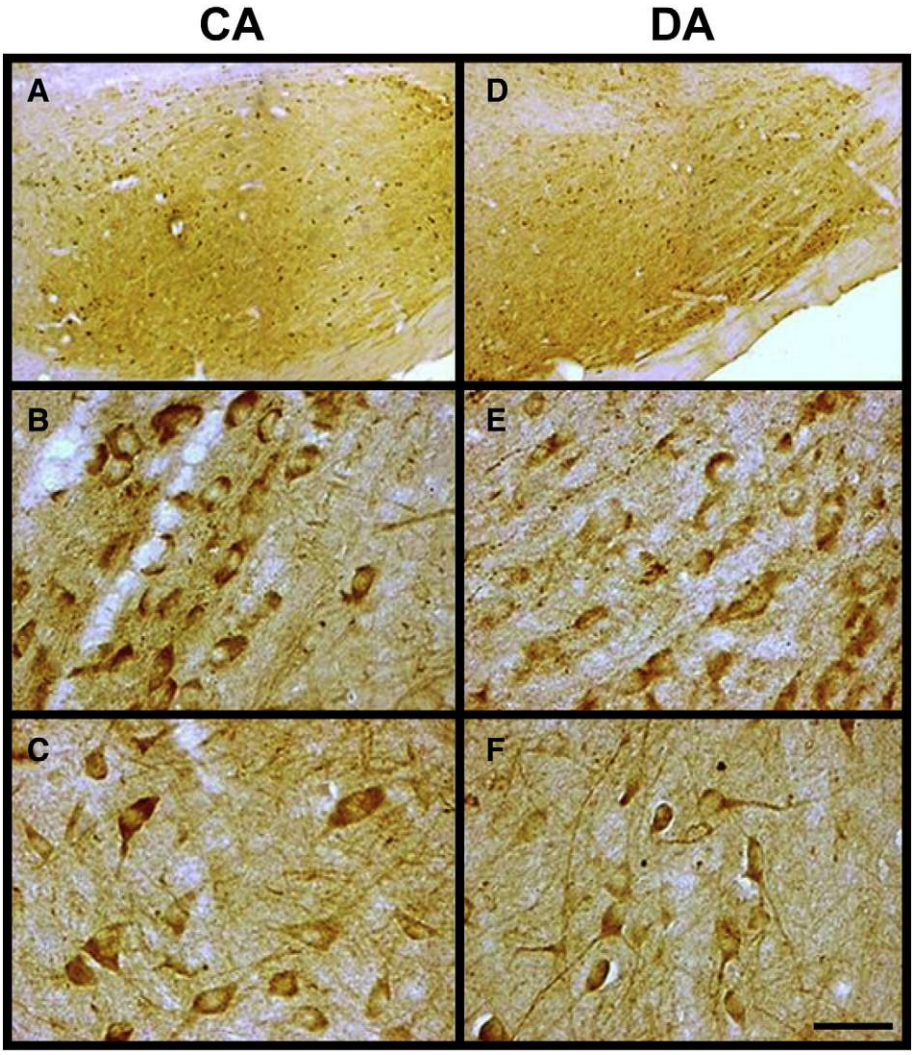

Fig. 4. Representative photomicrographs of BDNF-immunoreactive parasagittal sections at the mid-level of SN from adult rats fed control (CA) or EFA-restricted diet (DA). Low magnification images of CA (A) and DA (D) animals showing that BDNF-expressing cells are evenly distributed into the cytoarchitetonic limits of SN (bar = 250 μm; magnification power = 40×). High magnification images showing BDNF positive cells in the SNrm and SNcv from CA (B and C) and DA (E and F) animals (Scale bar = 30 μm; magnification power = 200×).

Conversely, DHA deficiency induces microglia activation in the striatum [60]. Considering that inducible NOS (iNOS) activity can be triggered in activated microglia under neurodegenerative conditions, we cannot discard the possibility that the high NO concentration observed in the striatum of our adult deficient animals could be, at least in part, due to this type of glial cell reactivity. It is well established that an excessive amount of NO can lead to the formation of peroxynitrite and other reactive nitrogenous species which can nitrate tyrosines of proteins modifying their structure and function, leading to cell death [61]. Thus, it is possible that this type of deleterious effect of NO could be involved in the significant reduction in both t-SOD and catalase enzymatic activities observed in the striatum of our omega-3 deficient animals.

4.2. Reduced striatum's resilience induced by chronic DHA deficiency until adulthood increases vulnerability of SNcv dopaminergic cell population

Gomes et al., [57] demonstrated that dopaminergic cells located in the ventral region of the rat's SN were more affected by 6-OHDA-induced oxidative stress in the striatum than other SN dopaminergic cell populations. In the present study, dopaminergic cell loss in the SNcv was detected in DA group, when oxidative stress was also seen in the striatum. A distinct neurochemical profile related to increased expression of genes encoding pro-inflammatory cytokines and decreased expression of several glutathione-related genes [62] has been described for this SN dopaminergic cell population. Interestingly, we did not find modifications in the number of these dopaminergic cells in deficient young animals, compared to respective controls [16]. Therefore, our findings in omega-3 deficient adult animals suggest vulnerability of this dopaminergic cell population to conditions of increasing oxidative stress, especially when the striatum is affected [57].

4.3. Omega-3 fatty acid availability affects the number of BDNF expressing cells in the SN

We hypothesized that another potential mechanism involved in SN neurodegeneration induced by midbrain DHA depletion could be a lower number of BDNF positive neurons in the SN. BDNF is believed to act as a paracrine/autocrine neurotrophic factor for dopaminergic and non-dopaminergic neurons of nigrostriatal system [23,24,63]. Evidence of post mortem studies in humans have indicated that loss of BDNF-expressing neurons in the SN may compromise their surviving neighbors, reducing the amount of their BDNF mRNA [31]. In the mouse's SN, BDNF is present in dopaminergic, GABAergic and nNOS positive neurons as well as in astrocytes [25]. A positive influence of DHA supplementation on BDNF levels and/or activity of its receptor TrkB has been reported in the hippocampus, cerebral cortex [20,21], spinal cord

Table 5
Estimated individual unilateral planimetric volumes of substantia nigra and respective total BDNF positive cell numbers in control (CA) and deficient (DA) groups.

Subjects	Thickness	Volume (mm^3)	CE	Total cells	CE
CA group					
C1	16.7	1.5	0.13	18,717	0.061
C2	21.9	1.2	0.13	17,786	0.072
C3	14.5	1.4	0.15	19,278	0.07
C4	16	1.5	0.12	19,743	0.062
Mean	17.2	1.4	0.132	18,881.0	0.066
SD	3.2	0.14	0.0125	841.9	0.006
CV^2	0.03	0.01		0.002	
CE^2				0.04356	
CE^2/CV^2				2.178	
CVB^2				−0.04156	
CVB^2 (%CV^2)				−2078%	
DA group					
D1	14.3	1.4	0.15	18,148	0.058
D2	15.9	1.4	0.14	16,539	0.06
D3	17.2	1.4	0.14	12,877	0.072
D4	13.8	1.3	0.13	14,015	0.06
Mean	15.3	1.375	0.140	15,394.7#	0.062
SD	1.6	0.05	0.0081	2389.7	0.006
CV^2	0.01	0.001	0.15	0.02409	0.010
CE^2				0.003844	
CE^2/CV^2				0.1595	
CVB^2				0.02024	
CVB^2(%CV^2)				84.04%	

CE, Scheaffer coefficient of error; CV, coefficient of variation; CVB, biological coefficient of variation; $CVB^2 = CV^2 - CE^2$; SD, standard deviation.
# $P < 0.05$ versus control.

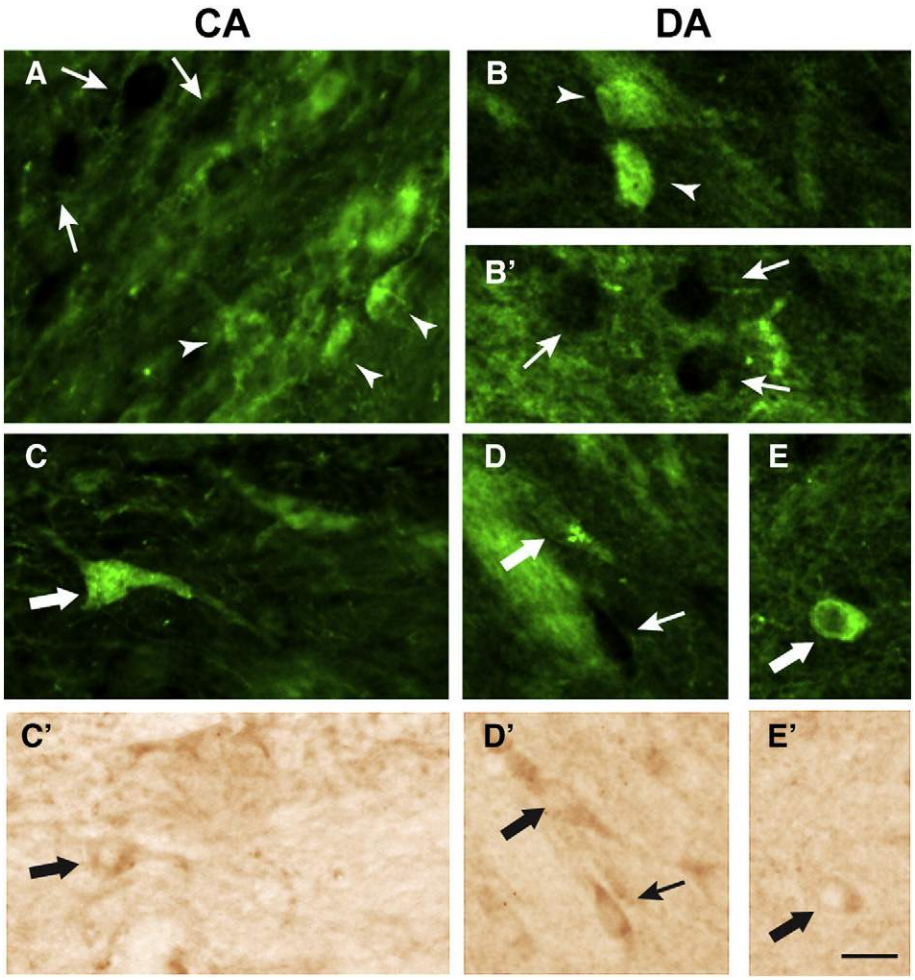

Fig. 5. Photomicrographs of epifluorescence microscopy showing SN sections immunoreactive for BDNF stained with DAB, and for TH visualized with DyLight-conjugated 488-IgG. As can be seen, examples of BDNF (thin arrows in brown or fluorescent images) or TH (green, arrowheads) single labeled cells are detected in the SN of rats fed control (A) or EFA-restricted (B and B′) diets. Double labeled cells for BDNF and TH are seen either in the control (C and C′, thick arrow) or in deficient animals (D and D′; E and E′, thick arrows). Thus, remaining TH-positive cells in the SN of DA group comprise a heterogeneous cell population regarding BDNF expression. Scale bar = 20 μm; magnification power = 400×.

[22] and in the striatum [55]. Conversely, dietary omega-3 fatty acid deficiency for a short-term or over two generations was able to reduce mRNA and/or protein levels of BDNF in the rat's cerebral cortex [64,65] and mouse's striatum [66]. To our knowledge, no previous study has investigated the repercussion of low levels of DHA on BDNF expression in the SN. In the present work, we demonstrated that the number of BDNF positive cells was ~20% lower in the SN of DA group, compared to the control. While this at first appears to be consistent with the loss of nigral dopaminergic neurons, double labeling experiments demonstrated that several surviving TH-immunoreactive cells also expressed BDNF in their soma and dendritic processes while in other remaining TH-positive cells, BDNF immunoreactivity was not detected. It has been demonstrated that BDNF expression in midbrain slice cultures can be enhanced by agonists of retinoid acid receptors (RAR) whose oral administration prevents dopaminergic cell loss induced by neuroinflammation in the SN [67]. DHA as well as AA are ligands for the retinoid X receptor [68] which, together with RAR, play diverse roles in brain development including morphological differentiation of dopaminergic neurons [69]. BDNF is also a direct target gene of the transcription factor Nurr1 [70] which is involved in the genesis, development and function of dopaminergic cells [71]. Thus, it is possible to speculate that the reduced number of BDNF-expressing cells and the lower values of cell body area observed in SN dopaminergic cell populations of DA animals could be a consequence of several cellular and molecular mechanisms impaired under conditions of chronic DHA deficiency.

5. Conclusion

In conclusion, our present findings demonstrate, for the first time, that distinct mechanisms of oxidative stress can be induced by EFA dietary restriction in the SN and the striatum, especially those involving NO production. Moreover, the data corroborated the hypothesis that an increasing DHA depletion until adulthood could reduce the striatum's resilience and the number of SN's BDNF-expressing cells, worsening the SN's dopaminergic cell loss. Taken together, our results reinforce the idea that EFA dietary restriction during brain development and maturation can modify the brain's competence to maintain suitable homeostatic responses under challenging conditions during adult life.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.12.023.

Conflict of interest

There is no conflict of interest in the work reported in the present paper.

Acknowledgements

The authors are grateful to Zenira Cosme Xavier for technical assistance and to Sidney Pratt and John David Bousfield for suggestions and reviewing the English version of the text. The advice of Dr. Cristovam Guerreiro Diniz and Dr. Roseane Borner in using Stereo Investigator Software is gratefully appreciated. The acquisition of the reagents used in this work was supported by the Brazilian National Research Council (CNPq); CAPES (PROCAD # 0008052/2006 and PROCAD NF-2009); and FACEPE (APQ 0036-2.07/11). We are also grateful to FACEPE which provided scholarships for Henriqueta Dias Cardoso, David Filipe de Santana and Catarina Gonçalves-Pimentel (DCR 0079-2.07/10).

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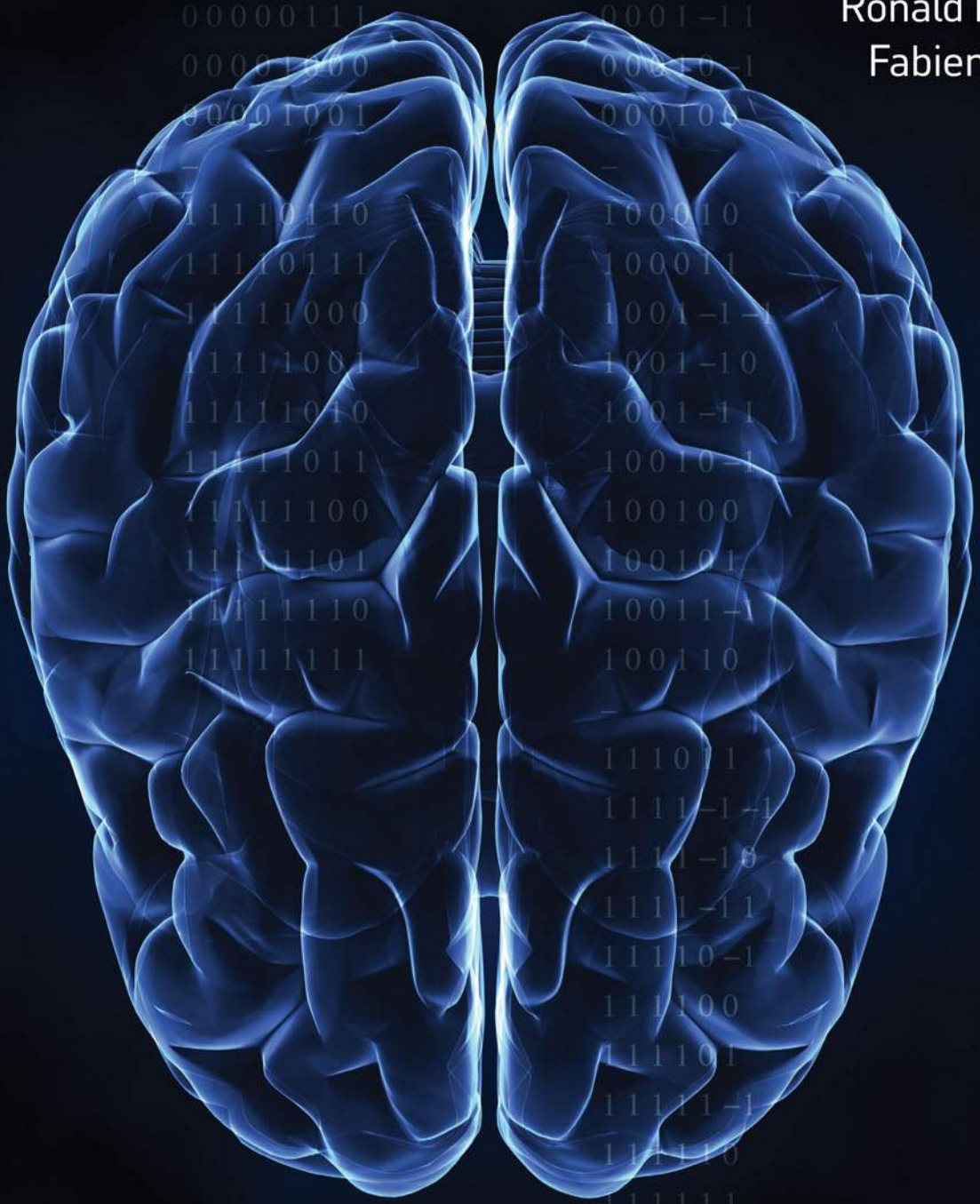
APÊNDICES

APÊNDICE B - Capítulo publicado no livro "Omega-3 Fatty Acids in Brain and Neurological Health": Substantia Nigra Modulation by Essential Fatty Acids

Omega-3 Fatty Acids in Brain and Neurological Health

Edited by
Ronald Ross Watson
Fabien De Meester



AP

OMEGA-3 FATTY ACIDS IN BRAIN AND NEUROLOGICAL HEALTH

OMEGA-3 FATTY ACIDS IN BRAIN AND NEUROLOGICAL HEALTH

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Academic Press is an imprint of Elsevier

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32 Jamestown Road, London NW1 7BY, UK
225 Wyman Street, Waltham, MA 02451, USA
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-410527-0

Typeset by MPS Limited, Chennai, India
www.adi-mps.com

Printed and bound in United States of America

14 15 16 17 18 10 9 8 7 6 5 4 3 2 1

CHAPTER

20

Substantia Nigra Modulation by Essential Fatty Acids

Belmira Lara da Silveira Andrade da Costa, Priscila Pereira Passos, Henriqueta Dias Cardoso, Catarina Gonçalves-Pimentel, Eraldo Fonseca dos Santos Junior, Juliana Maria Carrazzone Borba and Rubem Carlos Araújo Guedes

IMPORTANCE OF ESSENTIAL FATTY ACIDS AS NEUROPROTECTORS DURING BRAIN DEVELOPMENT AND AGING

A growing body of experimental and clinical evidence has indicated the importance of long-chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA) and arachidonic acid (AA), derived from the essential fatty acids (EFA) α-linolenic acid (ALA) and linoleic acid (LA), respectively, as critical modulators of brain function (reviewed in Uauy and Dangour, 2006; Innis, 2007; Zhang et al., 2011).

From an evolutionary point of view, it has been discussed that the rapid expansion of gray matter in the cerebral cortex coincided with the inclusion of nutrients from coastal seafood and other sources from inland freshwater containing high levels of DHA in the human diet (Crawford et al., 1999, 2001; Broadhurst et al., 2002; Bradbury, 2011). As early as the 1920s some researchers had already described the importance of EFAs, observing signs of dermal changes in rats and neurological and visual disorders in humans subjected to fat restriction in their diets (Burr and Burr, 1929). Since then, several functional aspects related to EFAs have been studied. An expressive increase in the number of studies on the importance of their balanced levels in the diet has been published, considering that DHA and AA can exert opposite effects on brain metabolism (Schmitz and Ecker, 2008; Bradbury, 2011).

During the growth spurt period, coincident with later stages of gestation and whole lactation period, there is a significant accumulation of all fatty acids in the brain (Green and Yavin, 1993; Martinez and Mougan, 1998; Green et al., 1999). Saturated, monounsaturated, and polyunsaturated fatty acids are differently distributed between the gray and white matters and among brain regions (Xiao et al., 2005; Levant et al., 2006). Their accretion occurs at distinct stages of brain development (Burdge and Postle, 1995; Green and Yavin, 1998; Green et al., 1999; Uauy and Dangour 2006; Innis, 2007). During pregnancy, the need for the LCPUFA is higher (Koletzko et al., 2008). They need to be shared among the various maternal tissues and the fetus according to their availability in the diet and their metabolism in the liver (Frazer and Huggett, 1970; Rapoport et al., 2007). As ligands for the retinoid X receptor, AA and DHA participate in diverse neurodevelopmental steps, including neurogenesis, morphological differentiation of some neurons and activity-dependent plasticity (Castro et al., 2001; Lengqvist et al., 2004). *In vitro* studies have also shown that DHA stimulates cell-cycle exit in retinal neuroprogenitor cells (Insua et al., 2003) and glial cell maturation (Joardar and Das, 2007; Joardar et al., 2006). Recently, it was also demonstrated that DHA promotes dopaminergic differentiation in induced pluripotent stem cells and inhibits teratoma formation in rats with Parkinson-like pathology (Chang et al., 2012).

Early and recent studies using *in situ* hybridization or microarray analysis have shown that DHA is able to regulate the transcription of many genes related to cell metabolism, cell signaling including the oxidative stress response, cell division, outgrowth and apoptosis

Omega-3 Fatty Acids in Brain and Neurological Health.
DOI: http://dx.doi.org/10.1016/B978-0-12-410527-0.00020-X

(Sessler and Ntambi, 1998; Berger et al., 2002; Kitajka et al., 2004; Lapillonne et al., 2004). In line with these findings, accumulating evidence has indicated that LCPUFA may act as neuroprotectors in the brain. DHA has been implicated in reducing apoptosis and necrosis in different classes of neurons (Lang-Lazdunski et al., 2003; Kim et al., 2010), and the inflammatory and anti-neurogenic functions of activated microglial cells (Ajmone-Cat et al., 2012) while AA can act as a neurotrophic factor on sensory neurons (Robson et al., 2010).

Some of the neuroprotective effects of DHA involve sub-products of its metabolism called docosanoids, especially neuroprotectin D1 (NPD1). Under conditions of inflammation and/or oxidative stress, NPD1 is able to reduce pro-apoptotic agents such as caspase-3, Bad and Bax, as well as to inhibit cyclooxigenase-2 activation and the pro-inflammatory factor NF-kB (Marcheselli et al., 2003; Chen and Bazan, 2005; Bazan et al., 2011; Mukherjee et al., 2004). Recent evidence has also shown that an ethanolamide derivative of DHA, called N-docosahexaenoylethanolamide (DEA) is a mediator of the DHA-induced increase in neurite outgrowth and synaptogenesis in hippocampal neurons (Kim et al., 2011a,b). A beneficial effect of the LA derivative 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA) on oxidative stress-induced neuronal death has been also reported (Yaguchi et al., 2010).

Taken together, these findings suggest that imbalance in AA/DHA levels early in life, and especially DHA deficiency, induce neurodegeneration (Yavin, 2006; Schmitz and Ecker, 2008; Bazan, 2006; Bazan et al., 2011). However, under physiological conditions, LCPUFA levels in brain membrane phospholipids decrease with aging (Guisto et al., 2002; Uauy and Dangour, 2006) and for this reason their role in healthy brain aging has been widely debated in the literature (reviewed in Zhang et al., 2011).

The extent and consequences of dietary omega-3 or omega-6 fatty acid deficiency on the brain are not yet completely understood. Nevertheless, it has been proposed that such deficiency can either contribute to the etiology of some neurodegenerative diseases (McNamara and Carlson, 2006; Heinrichs, 2010; Zhang et al., 2011) or worsens the age-induced modifications in neuron and glial cell reactivity that increases cell vulnerability to lesions (Latour et al., 2013). Therefore, dietary supplementation of LCPUFA, especially those of the omega-3 family, has been indicated as a potential therapeutic strategy as nutraceuticals, to reduce the risk of certain dopamine-associated neurological disorders (Chen et al., 2003; de Lau et al., 2005; Chao et al., 2012).

This section focuses on experimental data which reinforces the modulatory effect of EFAs on midbrain dopamine systems with special emphasis in the nigrostriatal system of basal ganglia circuitry.

SUBSTANTIA NIGRA VULNERABILITY TO NEURODEGENERATION

The basal ganglia are important components of the forebrain circuitry which fulfill cognitive, limbic, motor and learning functions (Graybiel et al., 1994; Hikosaka et al., 2000; Yin and Knowlton, 2006; Kreitzer and Malenka, 2008; Surmeier et al., 2011a). In this circuitry, the corpus striatum is the largest and the primary input nucleus that integrates afferent information from several regions of the cerebral cortex, the thalamus and dopaminergic and GABAergic innervation from the midbrain (Surmeier et al., 2011b).

Using interconnected closed or open neuronal loops with prefrontal, limbic, sensory and motor cortex, the basal ganglia network displays activity-dependent synaptic plasticity and coordinates action plans according to the motivation and motor information, and represents a neural substrate for procedural memory (revised in Graybiel, 2004; Kreitzer and Malenka, 2008; Haber and Calzavara, 2009; Pennartz et al., 2009). The dorsal striatum is especially involved in motor control while the ventral striatum, including nucleus accumbens (NAc), is mainly related to limbic and cognitive functions (Nicola, 2007). In this context, midbrain dopaminergic neurons from the substantia nigra and the ventral tegmental area (VTA) exert crucial roles modulating short-term cellular excitability as well as long-term changes in synaptic strength that shape network activity into the striatum and other basal ganglia nuclei (Joel and Weine, 1994; Surmeier et al., 2007; Kreitzer and Malenka, 2008).

Dopamine neurons from the substantia nigra pars compacta project mainly to the dorsal striatum characterizing the nigrostriatal or mesostriatal system. The dopamine mesocorticolimbic system that originated from the VTA can be subdivided into two pathways: one starts in the medial posterior part of VTA and projects to the medial prefrontal cortex, the basolateral amygdala and the core and medial shell of NAc; the second one originates from lateral portions of the VTA and the medial part of the substantia nigra projecting to the lateral shell of the NAc (Lammel et al., 2008). Thus, modifications in these dopaminergic systems underlie changes in movement and thought in some neurological disorders such as Parkinson's, Huntington's and Gilles de la Tourette's diseases (Albin et al., 1989; Graybiel, 2000; Wichmann et al., 2007) as well as in a number of psychiatric disorders such as schizophrenia

(van Kammen et al., 1989) and obsessive compulsive disorder (Aouizerate et al., 2004).

The vulnerability of substantia nigra dopamine neurons to lesions has been widely debated in the literature especially considering that it is a multifactorial process, involving extrinsic risk factors related to the substantia nigra environment and interconnected brain regions, and intrinsic factors related to cell metabolism and neuron-glia interaction (Hassler, 1938; Duke et al., 2007; González-Hernández et al., 2010; Bolam and Pissadaki, 2012; Sulzer and Surmeier, 2013; Shao et al., 2013). Compared to other midbrain dopaminergic nuclei such as VTA, retrorubral field and interfascicular nucleus or other basal ganglia nuclei, the substantia nigra has unique biochemical properties which render it particularly vulnerable to oxidative stress such as high iron content, low levels of endogenous antioxidant resource such as glutathione, catalase (CAT), and peroxidase enzymes (reviewed in Kidd, 2000; González-Hernández et al., 2010). Studies on rat or human substantia nigra have also indicated a progressive decrease in the activity of some antioxidant enzymes including superoxide dismutase (SOD) and CAT during physiological brain aging (Kolosova et al., 2003; Venkateshappa et al., 2012) which worsen its selective vulnerability to oxidative stress.

A high concentration of microglia has also been reported in the substantia nigra (Lawson et al., 1990). In conditions of mitochondrial dysfunction (Madathil et al., 2013) or lipopolysaccharide-induced insult (Arimoto and Bing, 2003; Arimoto et al., 2007), activated microglia release cytokines and free radicals such as superoxide radicals and nitric oxide (NO) (Minghetti et al., 1999; Duncan and Heales, 2005) which renders substantia nigra susceptible to develop neuroinflammation (reviewed in González-Hernández et al., 2010). The presence of receptors for pro-inflammatory cytokines has been shown in substantia nigra dopaminergic cells and especially in Parkinson's disease patients or experimental models of this disease, reinforcing their sensitivity to lesion under this stressful condition (Boka et al., 1994; Mogi et al., 2000; Ferger et al., 2004; Lofrumento et al., 2011).

As cell intrinsic risk factors, it has been well established in the literature that the dopamine (DA) metabolism *per se* contributes to the selective vulnerability of the substantia nigra. Dopaminergic cells possess a distinct physiology intrinsically associated with increased production of reactive oxygen species via metabolization by monoamino oxidase or auto-oxidation (Meiser et al., 2013). In these metabolic pathways, dopamine either generates hydrogen peroxide or it is converted into reactive quinones and superoxide anion. The latter can react with nitrogen reactive species producing peroxynitrite (Meiser et al., 2013). Evidence in mouse has also indicated that lower mitochondria content is found in dopaminergic compared to other non-dopaminergic cell populations present in the substantia nigra or in other midbrain dopaminergic nuclei such as VTA and interfascicular nucleus (Liang et al., 2007). Other studies have shown a redox modulation on the activity and expression of the tyrosine hydroxylase (TH), which represents the rate-limiting enzyme in the biosynthesis of DA (Di Giovanni et al., 2012). Structural risk factors contributing to dopamine cell vulnerability include unmyelinated or poorly myelinated long, highly branched axons and terminal fields. Physiological risk factors are pacemaker activity and broad action potentials. These features demand high energy and can be impaired under sustained conditions of oxidative stress or neuroinflammation (Bolam and Pissadaki, 2012; Sulzer and Surmeier, 2013). Besides the singular electrical properties, modifications induced by oxidative stress in some ion channels present in nigrostriatal dopaminergic neurons have been investigated as potential mechanisms involved in their selective demise in Parkinson's disease (Michell et al, 2007; Liss and Roeper, 2010). Moreover, it has been shown that maintaining L-type Ca^{2+} channels open in substantia nigra compacta DA neurons to keep pacemaker activity creates a basal mitochondrial oxidant stress. Epidemiological data also supports a linkage between L-type Ca^{2+} channels and the risk of developing Parkinson's disease (reviewed in Surmeier et al., 2011b).

SUBSTANTIA NIGRA DOPAMINE CELL POPULATIONS DISPLAY DIFFERENTIAL VULNERABILITY TO LESIONS

Despite the general features related to the substantia nigra environment and cell metabolism described above, dopamine cell populations located in the rostro-dorso-medial (SNrm) and caudo-ventro-lateral (SNcv) regions of this nucleus are not homogeneous. They rather differ in aspects related to their ontogeny, morphological and neurochemical profiles (Bayer et al., 1995; González-Hernández et al., 2004; Duke et al., 2007). Their projections to the corpus striatum are segregated into distinct functional divisions (Joel and Weiner, 2000; Prensa and Parent, 2001) and they also differ on their susceptibility to degeneration in Parkinson's disease in humans (Damier et al., 1999; Duke et al., 2007) and in rodent models (Rodríguez et al., 2001). DA cells located in the SNcv are usually more vulnerable to lesions compared to those of the SNrm. Several potential mechanisms involved in such

differential vulnerability have been proposed: expression of calcium binding proteins (Yamada et al., 1990; Gaspar et al., 1994) associated or not with the homeodomain transcription factor Pitx3 (Luk et al., 2013); availability of the glial cell line-derived neurotrophic factor (GDNF) from the striatum (Barroso-Chinea et al., 2005), levels of plasma membrane dopamine transporter (DAT) (Uhl et al., 1994; González-Hernández et al., 2004) and the transient down-regulation of DAT glycosylated form after dopamine cell loss (Afonso-Oramas et al., 2010). Increased expression of genes encoding pro-inflammatory cytokines and subunits of the mitochondrial electron transport chain and decreased expression of several glutathione-related genes have been found in the SNcv, compared to SNrm (Duke et al., 2007). Considering that most of these genes are particularly expressed in glial cells, one hypothesis was also raised that the nature of the selective sensitivity of SNcv dopamine neurons to adverse conditions could involve a distinct relationship among these cells, their environment and glial cells (Duke et al., 2007).

REPERCUSSION OF EFA DEFICIENCY OR SUPPLEMENTATION ON MIDBRAIN DOPAMINERGIC SYSTEMS

Several studies have indicated that serotonergic, mesostriatal and mesocorticolimbic dopamine systems can be particularly affected when brain DHA availability is reduced (review in Chalon, 2006). Using an animal model deficient in ALA over three generations, Delion et al. (1994) demonstrated that DHA deficiency was able to induce a significantly higher serotonergic 5-HT2 receptor density and a reduction in dopamine specific binding to receptor D2, associated with lower endogenous dopamine level in the frontal cortex of adult rats. However, in this study, none of these modifications were seen in the striatum. Deficiency of ALA for three generations also impaired dopamine vesicular release in the NAc and frontal cortex (Zimmer et al., 1998) and reduced the density of synaptic vesicles containing dopamine in this cortex (Zimmer et al., 2000a). In contrast, increased basal dopamine release and density of D2 dopamine receptors were observed in the NAc (Zimmer et al., 2000b). The vesicular monoamine transporter 2 (VMAT2) and D2 dopamine receptor mRNA levels were differentially altered in the dopamine mesolimbic and mesocortical systems of omega-3 deficient rats (Zimmer et al., 2002). In piglets, dietary EFA restriction for only 18 days from birth was also able to induce lower levels of dopamine, serotonin and norepinephrine in the frontal cortex (de la Presa Owens and Innis, 1999) indicating the sensitivity of these systems to a very short dietary treatment.

Studies on the reversibility of omega-3 LCPUFA deficiency-induced changes in dopaminergic neurotransmission demonstrated that even after restitution of an adequate diet after weaning, the stimulated release of dopamine in the NAc and frontal cortex, and the VMAT2 binding sites in the NAc did not recover completely (Kodas et al., 2002). Rats fed for 21 months with *trans* isomers of ALA showed reduced levels of endogenous dopamine in the frontal cortex, striatum and hippocampus. However, subsequent dietary supplementation with *cis* ALA was able to recover the dopamine concentration only in the frontal cortex (Acar et al., 2003). Other studies showed that, even when rats were fed from conception on a diet that produces a relatively modest decrease in brain DHA content (~20%), modifications in adult behavior indicative of dopaminergic dysfunction, such as basal and amphetamine-stimulated locomotor activity were found. Nonetheless, changes in catalepsy induced by haloperidol were reversed by supplementation of the diet at weaning (Levant et al., 2004). An extensive analysis using microarray, Western blot and immunohistochemistry was carried out by Kuperstein et al. (2005) in several brain regions of 2 week-old pups whose mothers were fed an α-linolenic deficient diet from conception. According to this study, a widespread increase in D1 and D2 dopamine receptors throughout the brain was found, including the substantia nigra and striatum, as well as several nuclei of the mesocorticolimbic system, such as VTA, NAc, amygdala, hippocampus, septum, thalamus, and frontal cortex.

Taken together, these findings have been discussed as potential modifications related to a behavioral hypersensitivity caused by impairment in DA production, consistent with the large spectrum of behavioral and cognitive modifications observed in omega-3 deficient animals (Reisbick and Neuringer, 1997; Wainwright et al., 1997; Carlson and Neuringer, 1999; Wainwright, 2002; Fedorova and Salem, 2006).

In line with the hypothesis of a deficient DA production, Ahmad et al. (2008), associating successive parity and ALA dietary restriction, reported fewer (~33%) TH-immunoreactive neurons in the substantia nigra pars compacta and VTA of omega-3 deficient adult animals as compared to animals fed an adequate diet. Moreover, a time-dependent decline was reported in TH mRNA and protein levels in the midbrain of lactating pups subjected to perinatal ALA deficiency (Kuperstein et al., 2008). The same authors also showed the time course of increasing dopamine receptor D1 and D2 mRNA expression in cerebral cortex and striatum, and D2 protein expression in the substantia nigra, septum, hippocampus, frontal cortex, amygdala and cerebellum, during the lactation period. In these same regions, no modification was detected in

the expression of dopamine plasma membrane transport (DAT), while a time-dependent decline was observed in the VMAT-2 protein and transcript levels in several brain regions related to midbrain dopamine system (Kuperstein et al., 2008).

Recently, Passos et al. (2012) investigated whether two functionally distinct dopamine cell populations of substantia nigra could be differentially affected by EFA dietary restriction over two generations. Considering their neurochemical profile and the distinct susceptibility to degeneration described above, it was hypothesized that dopaminergic cells located in the SNcv could be more vulnerable than those of SNrm to deleterious effects of an omega-3 deficiency. Using stereological assessment, the results did not corroborate the hypothesis, but paradoxically demonstrated for the first time a higher vulnerability of SNrm to the harmful effects induced by DHA depletion (~50%) in the midbrain of young animals. Although EFA dietary restriction affected the dopamine cell growth, assessed by body cell size in both SNrm and SNcv, significantly fewer TH-immunoreactive neurons (~20%) were found only in the SNrm compared to control conditions, consistent with reduced TH protein expression levels in the midbrain (Passos et al., 2012).

POTENTIAL MECHANISMS INVOLVED IN SUBSTANTIA NIGRA DOPAMINE CELL LOSS INDUCED BY EFA DIETARY RESTRICTION

The mechanisms involved in the substantia nigra dopamine cell loss induced by EFA dietary restrictions are not yet completely understood, but it seems that during brain development such deficiency could impair multiple homeostatic mechanisms that usually confer resistance to the dorsal tier of substantia nigra and even to the VTA, modifying the degeneration profile of midbrain dopaminergic cells.

Accumulating evidence has pointed to oxidative stress in the demise of dopamine cells as a relevant factor involved in the etiology and evolution of Parkinson's disease as well as in other neurodegenerative disorders (Thomas and Beal, 2007; Hashimoto and Hossain, 2011; Melo et al., 2011).

Accordingly, in experimental models of Parkinson's disease, it has been recently shown that the dietary supplementation of DHA may partially restore dopaminergic neurotransmission after 6-hydroxidopamine (6-OHDA)- or 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP)-induced striatal lesions which produce oxidative stress (Bousquet et al., 2008; Cansev et al., 2008). Moreover, DHA supplementation was able to increase the SOD activity in the corpus striatum (Sarsilmaz et al., 2003) as well as significantly decreased cyclooxigenase-2 activity and prostaglandin E2 levels in the substantia nigra, attenuating MPTP-induced dopaminergic cell death (Ozsoy et al., 2011).

Considering these previous studies, Cardoso et al. (2012) investigated whether EFA dietary restriction for one (F1) or two (F2) generations could induce lipoperoxidation or modify the antioxidant activity of SOD or CAT in the substantia nigra and striatum of rats. This study demonstrated that in first generation adult animals, this nutritional deficiency caused a 28% DHA depletion. Nevertheless, increased SOD enzymatic activity was observed in both regions, protecting them against lipoperoxidation. In young animals subjected to EFA deficiency for two generations, signals of degeneration in dopaminergic and non-dopaminergic neurons were associated with a significant increase in lipoperoxidation and decreases in the CAT enzymatic activity were detected in the substantia nigra. In contrast, a strong resilience of the striatum to this oxidative insult was observed, in spite of a similar level of DHA depletion (~50%) in both regions.

The results obtained by Cardoso et al. (2012) corroborated the hypothesis that oxidative stress in the substantia nigra could be one of the potential mechanisms involved in the dopamine cell loss induced by DHA deficiency (Ahmad et al., 2008; Passos et al., 2012). Moreover, they highlight the importance of DHA in maintaining the redox balance in the substantia nigra, reinforcing the protective action of DHA dietary supplementation on substantia nigra cell populations under oxidative stress conditions (Ozsoy et al., 2011).

As previously described in this chapter, studies on rat or human substantia nigra have indicated a progressive decrease in the activity of several antioxidant enzymes including SOD and CAT during physiological brain aging (Kolosova et al., 2003; Venkateshappa et al., 2012). Thus, higher levels of oxidative stress induced by DHA deficiency in the substantia nigra of young animals may accelerate the degenerative profile of this nucleus increasing the risk of dopamine-related diseases such as Parkinson's disease. Nevertheless, evidence from our laboratory has shown that the presence of oxidative stress depends on the magnitude of DHA depletion in the midbrain. Recent data from our group in F1 adult animals subjected to EFA dietary restriction from conception demonstrated that 28% DHA depletion in the midbrain is enough to induce dopamine cell loss in the SNrm, even in the absence of lipoperoxidation, previously demonstrated by Cardoso et al. (2012). Figure 20.1A shows results reported by Cardoso et al. (2012) demonstrating the absence of lipoperoxidation in F1 adult animals fed an EFA-deficient diet. Figure 20.1B shows representative images of brain parasaggital sections through substantia nigra in F1 control and

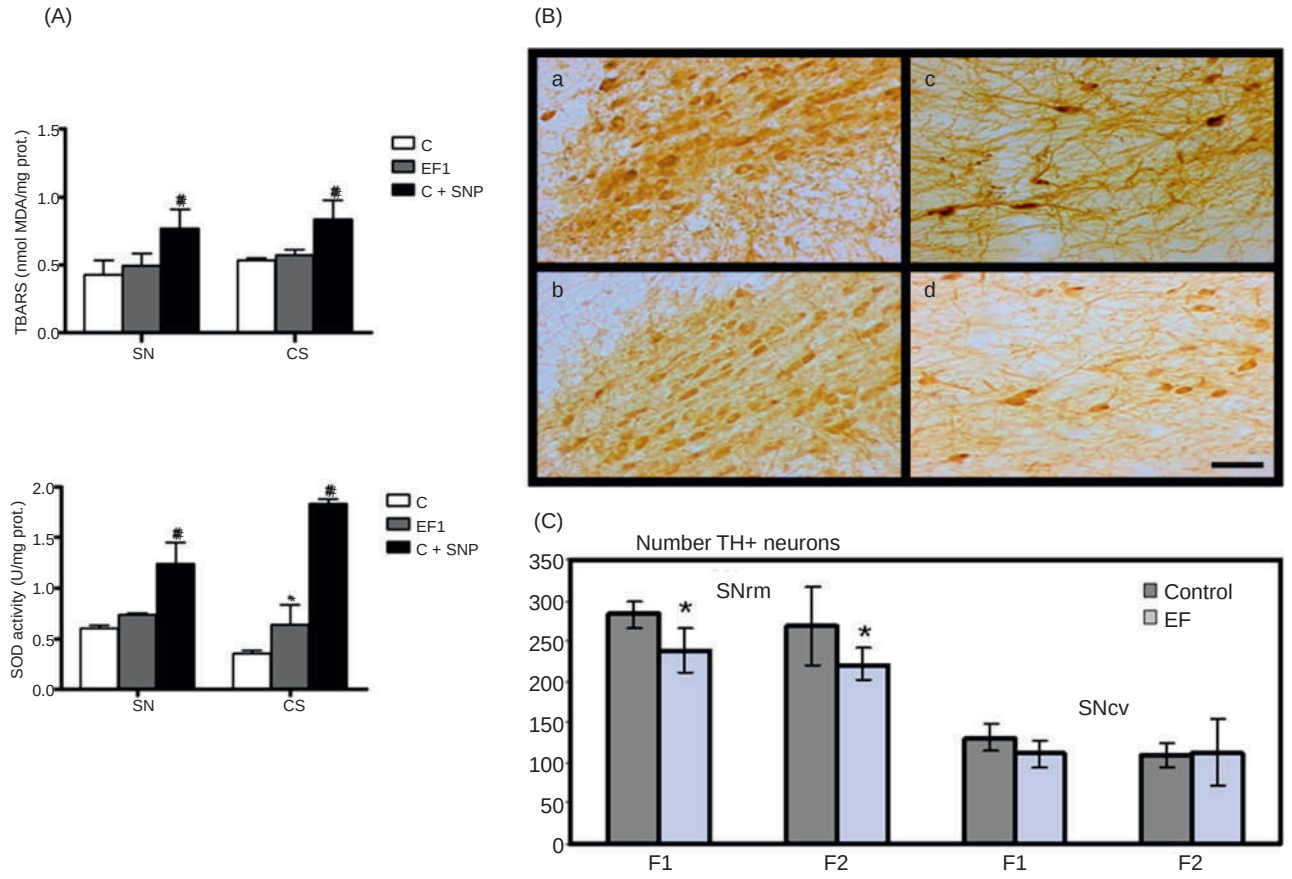

FIGURE 20.1 **Effect of EFA dietary restriction for one or two generations on oxidative stress markers in the nigrostriatal system and TH-immunoreactivity in the substantia nigra.** A: Thiobarbituric acid-reactant substances (TBARS) levels (A) and total superoxide dismutase (t-SOD) activities (B) in the pool of substantia nigra and corpus striatum from first generation adult rats fed essential fatty acid-restricted diet and controls ($n = 12$ per group). $^{*}P < 0.05$ compared to control group. Treatment of control homogenates with sodium nitroprusside (SNP) was used as a positive control in all the experiments.
$^{\#}P < 0.001$ compared to control or EF1groups. *Source*: Cardoso et al. (2012).
B: Representative photomicrographs of TH-immunoreactive parasagittal sections at the mid-level of substantia nigra from first generation adult rats fed control (C) or EFA-deficient diet (EF). Images of (A) and (B) show dopaminergic cells in the SNrm and SNcv from control group; (C) and (D) from EF1 group (bar = 30 μm).
C: Average number of TH positive neurons in the SNrm and SNcv of F1 adult and F2 young control (C) and EFA-deficient groups. Data were obtained in one parasagittal section at the mid-level of the substantia nigra and express mean ± SD. $^{*}p < 0.05$ compared to control group.

experimental animals. Figure 20.1C compares the number of TH-immunoreactive cells, at the mid-level of substantia nigra, between control and omega-3 deficient animals in F1 adult animals and F2 young animals in the rostro-dorso-medial (SNrm) and caudo-ventro-lateral (SNcv) regions. This bidimensional analysis in F2 young animals shows that the magnitude of SNrm dopamine cell loss (~20%) is similar to results previously reported by Passos et al. (2012) using stereological assessment.

The apparent striatum resilience to oxidative insult in F2 young animals subjected to EFA deficiency (Cardoso et al., 2012) is also occurring in the presence of other reactive processes in this nucleus. We have examined stereotyped behaviors such as licking, head bobbing, turning and jumping effects induced by acute administration of the dopaminergic agonist apomorphine (1 mg/Kg, i.p.) in F2 young animals (35–40 days; n = 11 per group). Figure 20.2 shows the increased degree of apomorphine-induced stereotypy in the experimental (EF2Y) group when compared to controls (C). Such behavioral analysis reinforces previous evidence that the expression of dopamine receptors in the nigrostriatal system can be modified by EFA dietary restriction.

It is worth noting that a relatively short-term feeding of an ALA-restricted diet was able to induce lower brain-derived neurotrophic factor (BDNF) levels in the mouse striatum (Miyazawa et al., 2010) and cerebral cortex (Rao et al., 2007). This sensitivity of striatum to changes in BDNF levels as a function of DHA concentration was also observed in other studies. Dietary

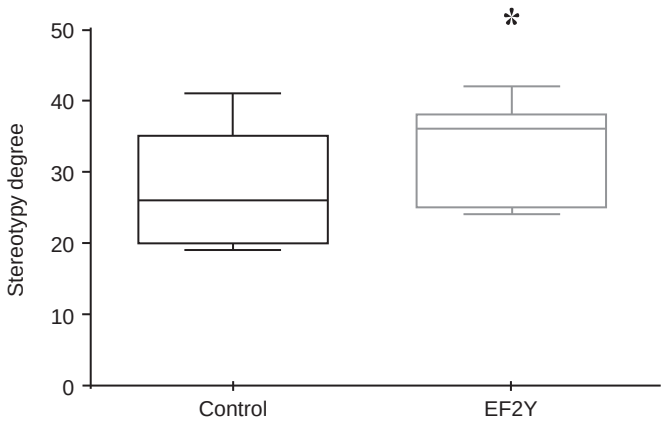

FIGURE 20.2 Effect of EFA dietary restriction over two generations on apomorphine-induced stereotypy. Apomorphine-induced stereotypy in young rats (30–45 days) fed the control (C) and EFA-deficient diet (EF2Y). Stereotyped behaviors such as licking, head bobbing, turning and jumping were measured following apomorphine (1 mg/kg, i.p.). * $p = 0.043$, Wilcoxon matched-pairs signed rank test.

DHA supplementation led to a strong reaction of the striatum under conditions of MPTP-induced oxidative stress, increasing BDNF content more than under control conditions (Bousquet et al., 2009). A stimulatory effect of DHA on BDNF expression has also been reported for other regions such as hippocampus, cerebral cortex and spinal cord (Vines et al., 2012; Ying et al., 2012) indicating a widespread effect that can occur in neurons with different neurochemical profiles.

BDNF is a potent dopaminergic neurotrophin produced in the substantia nigra (Hyman et al., 1991; Stahl et al., 2011) and transported to the striatum (Altar and Distefano, 1998). It is also a direct target gene of the transcription factor Nurr1 (Volpicelli et al., 2007) which is involved in the genesis, development, and function of dopaminergic cells (Jankovic et al., 2005). Another point to be considered is the fact that DHA supplementation can reduce the loss of the transcription factor Nurr1 in the substantia nigra under conditions of MPTP-mediated oxidative stress (Bousquet et al., 2009).

Early and recent studies support the hypothesis that reduced BDNF levels can be a potential mechanism involved in the DHA depletion-induced dopamine cell loss in the nigrostriatal system. Intrathecal infusion of BDNF reduced the loss of dopamine neurons and the severity of Parkinson's disease in MPTP treated monkeys (Tsukahara et al., 1995). Patients with Parkinson's disease showed reduced BDNF mRNA expression in the substantia nigra (Howells et al., 2000) and BDNF protein levels in the substantia nigra and striatum (Mogi et al., 1999). Furthermore, chronic deprivation of TrkB signaling led to selective late onset of nigrostriatal dopaminergic degeneration (Baydyuk et al., 2011)

It was recently reported that there is a modulatory effect of DHA on other neurotrophins with action in the nigrostriatal system. DHA supplementation was able to increase GDNF and neurturin levels in the substantia nigra, reducing MPTP-induced dopaminergic cell death (Tanriover et al., 2010). As shown by Barroso-Chinea et al. (2005), most dopaminergic neurons of SNrm and VTA, but not those of SNcv, contain GDNF retrogradely transported from the striatum. Thus, the GDNF reduction caused by DHA depletion cannot be disregarded as a potential mechanism involved in the differential vulnerability of the SNrm dopamine cells previously reported (Passos et al., 2012). Future studies should be carried out in order to address this issue. Figure 20.3 shows several effects induced by EFA dietary restriction on midbrain dopamine systems.

A modulatory effect of omega-3 LCPUFA upon neuroinflammation in the nigrostriatal system has recently been demonstrated. In the striatum of suckling pups subjected to perinatal ALA deficiency, microglia activation was reported by Kuperstein et al. (2008). Conversely, elevated dietary omega-3 LCPUFA levels were protective against dopaminergic damage associated with neuroinflammation in experimental models of Parkinson's disease. For example, dietary supplementation with ethyl-EPA was able to protect mice against MPTP-induced hypokinesia and other behavioral deficits, and prevented the increase in TNF-α and interleukins (Luchtman et al., 2012). Another recent study showed that rats fed a 15% fish oil diet for 2 weeks prior to injection of lipopolysaccharide in the substantia nigra were protected against dopaminergic cell loss, microglia activation, TNF-α and interleukin 1 expression (Ji et al., 2012). Although these are promising results, the mechanisms involved in such effects still deserve future investigation.

Glutamate excitotoxicity has also been implicated in the vulnerability of substantia nigra dopamine cells (reviewed in González-Hernández et al., 2010). Under physiological conditions, LCPUFA can exert multiple effects on the glutamatergic system of the cerebral cortex or hippocampus: some of them favor hyperexcitability (Miller et al., 1992; Nishikawa et al., 1994) while others can decrease synaptic glutamate transmission and increase neuroprotection (Vreugdenhil et al., 1996; Lauritzen et al., 2000). In the substantia nigra, experimental evidence has indicated that DHA and AA can play an important role in the modulation of neuronal excitability by reducing GABA and glycine response, and potentializing glutamatergic transmission via NMDA receptors (Hamano et al., 1996).

The substantia nigra receives glutamatergic afferent neurons from the pedunculopontine tegmental nucleus (PPTg) and subthalamic nucleus (Forster and Blaha, 2003). Stimulation of PPTg glutamatergic neurons with

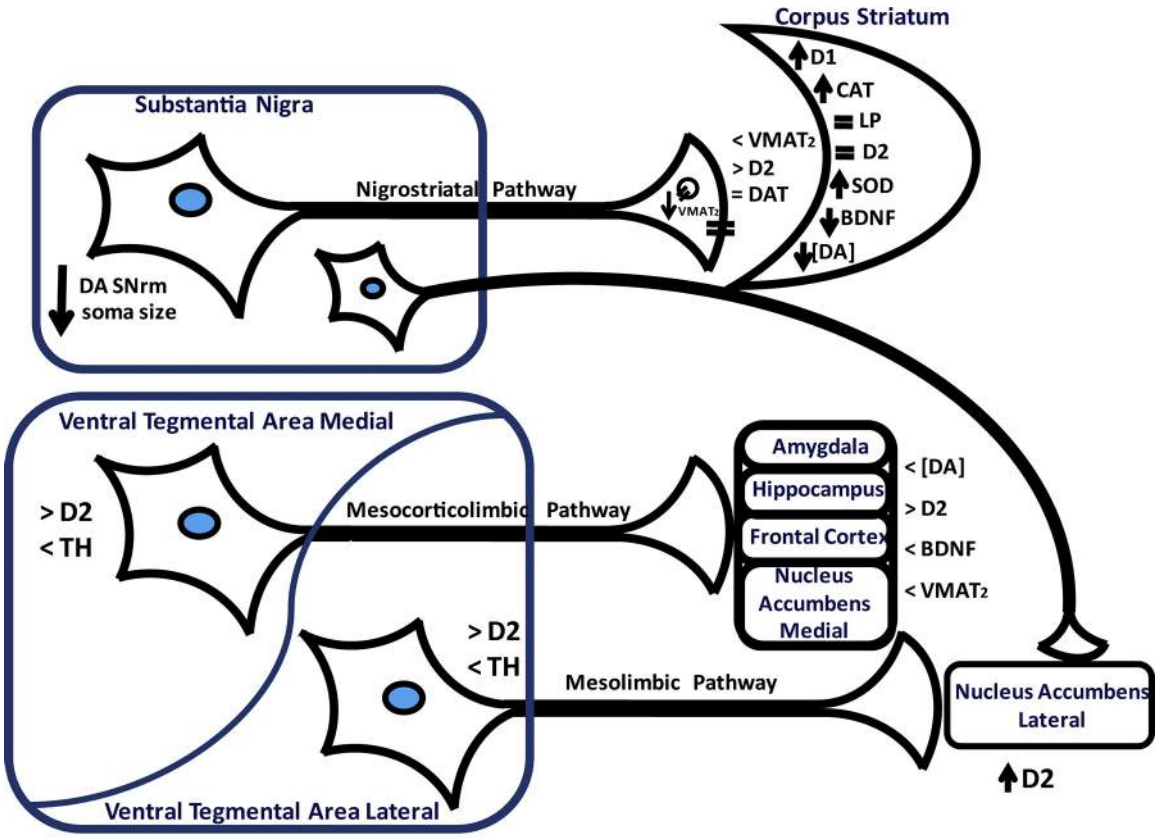

FIGURE 20.3 Schematic drawing illustrating neurochemical effects induced by EFA dietary restriction on midbrain dopamine systems. All data were obtained from literature cited in this chapter. [DA] = dopamine concentration; [D2] = dopamine receptor type 2 expression; [D1] = dopamine receptor type 1 expression [$VMAT_2$] = vesicular monoamine transporter 2 expression; CAT = catalase enzymatic activity; SOD = superoxide dismutase enzymatic activity; LP = lipoperoxidation; BDNF = brain-derived neurotrophic factor expression.

kainic acid was able to induce neurodegeneration in substantia nigra dopamine cells (Gonzalez-Hernandez et al., 1997). Although some experimental data indicate that glutamate might contribute to substantia nigra dopamine cell degeneration, the impact of omega-3 dietary deficiency or supplementation on this glutamatergic action into the substantia nigra is not yet completely understood. However, the repercussion of such deficiency during aging in the hippocampus has recently been investigated (Latour et al., 2013). According to these authors, reduced levels of DHA were able to worsen the age-induced degradation of glutamatergic transmission, modifying its astroglial regulation and glial cell proliferation.

Under some pathological conditions, increased levels of AA and DHA are synthesized and released from astrocytes, exerting modulatory actions on neuronal excitability and protection (Yoshida et al., 1980; Siesjö et al., 1982). A number of studies have demonstrated the importance of DHA on astrocyte differentiation and viability (Champeil-Potokar et al., 2004; Joardar and Das, 2007), distribution of connexin-43 gap junctions (Champeil-Potokar et al., 2006) and glutamatergic activity involving astrocytes (Berry et al., 2005; Grintal et al, 2009) in the cerebral cortex. Therefore, it remains to be shown how neuron–astrocyte cross-talk can be modulated by DHA deficiency or supplementation in the nigrostriatal system, considering the importance of astrocytes to this system (Shao et al., 2013).

In conclusion, current experimental data reinforces the idea that adequate EFA levels can act as important players for the tonic activity of midbrain dopamine systems. Providing adequate molecular signaling, these fatty acids seem to be necessary to modulate key functions such as dopamine metabolism, release and uptake; receptor affinity; redox balance; anti-inflammatory response; and neurotrophin synthesis. Taken together, these actions may improve the competence of this neuronal system to maintain a suitable resilience during development and brain maturation. Such experimental studies seem to be in agreement with prospective studies in humans that have positively associated a dietary intake of omega-3 PUFA with a lower risk of developing Parkinson's disease (Chen et al., 2003; de Lau et al., 2005).

Acknowledgments

The authors are grateful to the Brazilian National Research Council (CNPq); CAPES (PROCAD # 0008052/2006 and PROCAD NF-2009); FACEPE (APQ 0036-2.07/11). We are also grateful to FACEPE which provided scholarships for Henriqueta Dias Cardoso, Eraldo Fonseca dos Santos Junior and Catarina Gonçalves-Pimentel (DCR 0079-2.07/10).

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Further Reading

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APÊNDICES

APÊNDICE C - Artigo submetido a "European Journal of Neuroscience": A differential astrocyte reactivity is induced by omega-3 fatty acid deficiency in nuclei of rat basal ganglia

A differential astrocyte reactivity is induced by omega-3 fatty acid deficiency in nuclei of rat basal ganglia

Journal:	*European Journal of Neuroscience*
Manuscript ID:	Draft
Manuscript Type:	Research Report
Date Submitted by the Author:	n/a
Complete List of Authors:	Santana, David; Universidade Federal de Pernambuco, Physiology and Pharmacology Cardoso, Henriqueta; Universidade Federal de Pernambuco, Physiology and Pharmacology Pimentel, Catarina; Universidade Federal de Pernambuco, Physiology and Pharmacology Santos-Junior, Eraldo; Universidade Federal de Pernambuco, Physiology and Pharmacology Passos, Priscila; Universidade Federal de Pernambuco, Physiology and Pharmacology Guedes, Rubem; Universidade Federal de Pernambuco, Dept. of Nutrition Neto, Vivaldo; Universidade Federal do Rio de Janeiro, Dept. Anatomia Rodrigues, Marcelo; Universidade Federal de Pernambuco, Physiology and Pharmacology Andrade-da-Costa, Belmira Lara; Universidade Federal de Pernambuco, Physiology and Pharmacology
Key Words:	astrocyte, basal ganglia, Glial cell physiology, Rat striatum

To: Journal of European Neuroscience,
Section: Molecular & Synaptic Mechanisms

A differential astrocyte reactivity is induced by omega-3 fatty acid deficiency in nuclei of rat basal ganglia

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Running title: Omega-3 deficiency modify astroglial reactivity
Text pages: 29
Figures: 6
Tables: 2
The total number of words in:
the Whole manuscript: 8679
the Abstract: 250
the Introduction: 500

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Abstract

Omega-3 fatty acid (n-3) deficiency for two generations induces oxidative stress and neurodegeneration in the rat nigrostriatal system. To understand whether impairment in glial cell function contributes to this effect, this study assessed astrocyte morpho-functional parameters in both substantia nigra and striatum of young and adult rats. Animals were divided into two groups according to the diet, supplied from mating and over two generations. The lipid source of control diet was soybean and the n-3 deficient diet was coconut oil. Glutamine synthetase activity, GFAP isoforms expression, morphological changes and number of $GFAP^+$ astrocytes were analyzed. The results showed a significant increase in glutamine synthetase activity in the striatum of n-3 restricted young (2.5 fold) and adult (2.0 fold) animals in comparison to the control. In the substantia nigra, this increase was observed only at adulthood (4.0 fold) and astrocytes showed an increased arborization area; whereas in the striatum, a higher fractal dimension was also found at both ages. Lower number of astrocytes was found in the substantia nigra of n-3 deficient young (40% less) and adult (20% less) animals compared to control. The expression profile of GFAP isoforms showed that n-3 deficiency was associated with a decreased expression of the phosphorylated 50 kDa band, especially in the midbrain, where additional isoforms were detected through adulthood. The results show that the impact of n-3 dietary restriction on astrocytes is region specific within basal ganglia and indicate that their dysfunction in the substantia nigra may contribute to worsen neurodegeneration in this nucleus.

Keywords: astrocytes, docosahexaenoic acid, substantia nigra, striatum, glutamine synthetase

Introduction

It is well established that brain physiology involves a wide range of astrocyte adaptive functions (Parpura *et al.*, 2012) and that reduction in their activity is related to synaptic dysfunction and neuronal vulnerability to lesions (Zou *et al.*, 2010; Verkhratsky *et al.*, 2013).

Astrocytes in the nigro-striatal system secrete a number of neurotrophic factors (Lin *et al.*, 1993; Knott *et al.*, 2002; Petrova *et al.*, 2003) which have essential roles in the proliferation, differentiation and survival of dopaminergic cells (Mena *et al.*, 2002; Mena & Garcia de Yebenes, 2008). Moreover, astrocyte importance is noteworthy in the redox balance, considering that some of the inducible antioxidant systems are mainly expressed in these cells (Takuma *et al.*, 2004; Iitsuka *et al.*, 2012), providing neuroprotection, especially in the substantia nigra (SN) (Damier *et al.*, 1993; Makar *et al.*, 1994; Ishida *et al.*, 2006). Nevertheless, under conditions of intense neurodegeneration, astrocytes can release pro-inflammatory cytokines, suggesting that in this situation they may have deleterious effects on this nucleus (Kohutnicka *et al.*, 1998; Przedborski *et al.*, 2000).

Neuroprotective actions of astrocytes include also their ability to synthesize and release the n-3 and n-6 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA) and arachidonic acid (AA) into the extracellular medium (Moore, 2001). These PUFAs play important roles on neuronal excitability, vascular and astrocyte responses (Vreugdenhil *et al.*, 1996; Lauritzen *et al.*, 2000; Blondeau *et al.*, 2007). *In vitro* studies have demonstrated that DHA modulates the activity of glutamate transporters (Grintal *et al.*, 2009), increases the viability and stimulates the functional distribution of conexins in cerebral cortex astrocytes (Champeil-Potokar *et al.*, 2006). Conversely, treatment of astrocyte cultures with low DHA concentration impairs their differentiation and modify the expression profile of glial fibrilary acid protein (GFAP) isoforms (Joardar & Das, 2007).

Recent evidence *in vivo* reported that the DHA deficiency worsen harmful effects of aging in the hippocampus astroglia (Latour *et al.*, 2013).

We have recently shown that n-3 deficiency for two-generations induces neurodegeneration accompanied by increased lipoperoxidation (LP) levels and reduced anti-oxidant resources in the SN but not in the striatum of young rats, despite similar level (~50%) of DHA depletion (Cardoso *et al.*, 2012). Prolonging n-3 deficiency until adulthood reduced the DHA levels by 65%, affected BDNF expression in the SN and increased nitric oxide production in the striatum, reducing the resilience of this nucleus (Cardoso *et al.*, 2013). Taking into account these deleterious effects on redox balance, we hypothesized that n-3 dietary restriction might impair morpho-functional parameters involved in astroglial activity in the nigrostriatal system. We also tested the hypothesis that SN astrocytes would be more vulnerable to conditions of DHA depletion than those located in the striatum.

To address this question we first analyzed the activity of the glutamine synthetase enzyme which has important functions related to the glutamine-glutamate cycle involved in the regulation of energy metabolism by astrocytes (Pellerin *et al.*, 2007). Morphological changes, signs of proliferation and the expression profile of GFAP, were also analyzed as indicators of astrocyte phenotypic plasticity.

Materials and Methods

Animals and diets

Adult female Wistar rats weighing 250–300 g were fed from mating throughout pregnancy and lactation on control or experimental diet, both containing around 400 kcal/100 g and differing only in the lipid source. The composition of the diets and their fatty acids content are shown in Tables 1 and 2, respectively. The

diets were prepared according to (Soares *et al.*, 1995) submitted to transesterification according to (Hartman & Lago, 1973) and analyzed using gas chromatography.

The control diet contained 5% of soybean oil with adequate levels of saturated, monounsaturated, alpha-linolenic and linoleic fatty acids. The experimental diet contained 5% coconut oil with reduced levels of linoleic acid (~7 times) and alpha-linolenic acid (~12 times), and higher levels of saturated (2 fold) and monounsaturated (2.5 fold) fatty acids, compared to control. As previously described, this diet is commonly used to induce an n-3 deficiency in the central nervous system because, despite its reduced level of linoleic acid, AA levels are not affected and only DHA is significantly reduced in the brain (Ling *et al.*, 2010; Cardoso *et al.*, 2012; Passos *et al.*, 2012; Cardoso *et al.*, 2013). Using this diet over two generations we have shown that DHA depletion in the SN and striatum were similar and correspondent to 50% and 65% at young (30-40 days old) and adult (90-110 days old) rats, respectively (Cardoso *et al.*, 2012; Cardoso *et al.*, 2013).

At parturition, litter size, total litter weight and mean birth weight of the pups were recorded. Litters containing at most 13 pups were then reduced to 6 pups each, by keeping the median weighted animals. Dams and male pups were divided into two main groups according to the nutritional condition: control (C) and deficient rats (D). After weaning, on postnatal day 21, the rat pups were separated and fed *ad libitum* the same diet as their respective mothers until adulthood (90-110 days) when they were allowed to mate to provide the second generation groups, which were analyzed at young (30–42 days, CY and DY groups) and adult ages (90-110 days, CA and DA groups). In each group, male pups were sampled randomly from different litters, housed three per cage in a room maintained at 22° - 28°C with 67% relative air humidity and kept on a 12 h light/dark cycle (lights on at 6:00 h). A total of 108 males of both groups were the object of the present study. All animal procedures were carried out in accordance with the norms of the Ethics Committee

for Animal Research of Federal University of Pernambuco (CEEA), which specifically approved this study (protocol # 009428/200633), and complies with the ‘‘Principles of Laboratory Animal Care’’ (NIH, Bethesda, USA).

Please, insert Tables 1 and 2 around here

Protein extraction and Western blotting analysis

Each experimental day, six young or adult animals obtained from at least three litters per group were anesthetized with isoflurane and then decapitated. The preanesthesia with volatile agents to prevent pain and reflexes is recognized by animal welfare regulatory agencies (e.g. CONCEA, Brazil; UK Animals Scientific Procedure, 1986). According to (Davis, 1996) , isoflurane anesthesia prior rat decapitation does not induce significant effects on plasma and liver carnitine, plasma beta-hydroxybutyrate and free fatty acids, or liver free fatty acids, triglycerides, free Coenzime A, and acetyl coenzyme A. For this reason we adopted this procedure in the present study.

After decapitation, the regions containing the SN, cerebral cortex or striatum were rapidly dissected and placed in a 0,9% (w/v) NaCl solution at 2° C. Pooled tissue was obtained from each group and homogenized in a freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.5 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml leupeptin and 0.05% Triton X-100. An aliquot was taken to determine total protein content. Samples containing 1,5 mg of protein were diluted in a sample buffer (62.5 mM Tris/HCl, pH 7.4, 4% SDS, 10% glycerol, 10% mercaptoethanol and 0.002% bromophenol blue) and boiled for approximately 3 min. Fractioning of protein samples was achieved using 10% polyacrylamide gels containing 0.1% SDS. After separation, the protein bands were transferred from the gel onto a

nitrocellulose membrane, as described by Towbin et al. (1979). The nitrocellulose blots were incubated with rabbit anti-GFAP polyclonal antibody (1:500; Dako) overnight, at 4° C. They were subsequently exposed to HRP-conjugated goat anti-rabbit IgG as the secondary antibody (1:1000; Sigma-Aldrich, USA) for 2 h . The signals were detected using a 0.16% solution of 3- amino-9-ethylcarbazole in a 50 nM sodium acetate buffer (pH 5) containing 0.05% Tween-20 and 0.03% H_2O_2 or DAB Enhanced Liquid Substrate System (Sigma) according to the manufacturer's instructions. All the experiments were carried out three times using two different homogenates, each time. Thus, 12 animals per group were used.

Glutamine synthetase activity

For each experiment, three young or adult animals per group were anesthetized with isoflurane and then decapitated. The regions containing the SN or striatum were rapidly dissected and the pooled tissue of each group was homogenized in a 0.15 M KCl solution (1:5 w/v) for the measurement of GS activity (Petito *et al.*, 1992). SN or striatum homogenates (0.1 ml) were added to a reaction mixture containing 100 mM imidazole-HCl (pH 7.0) buffer, 50 mM glutamate, 50 mM hydroxylamine hydrochloride, 10 mM $MgCl_2$ and 10 mM ATP and 10 mM 2-mercaptoethanol, and incubated for 15 min at 37°C. The reaction was stopped by adding 0.4 ml of a solution containing 370 mM $FeCl_3$, 200 mM TCA, and 670 mM HCl. After centrifugation (2,000 rpm) the supernatant`s absorbance was read at 530 nm using a plate-reader and assessed against a standard curve of known concentrations of gamma-glutamyl hydroxamate. One unit was defined as the enzyme activity needed to form 1 mmol of gamma-glutamyl hydroxamate per minute. All experiments were carried out in triplicate and repeated at least twice. Results were plotted using GraphPad Prism Software, version 5.0 for Windows (San Diego, CA, USA) and the

statistical analysis was performed using ANOVA followed by Bonferroni as the post-hoc test in the OriginPro 8 Software (Northamptom, MA, USA).

Immunohistochemical procedure

Rats (seven animals per group) were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially first with saline (0.9% NaCl; 50–100 ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 (200-400 ml). Perfusion was always performed between 12:00 and 18:00, using a continuous infusion pump (Harvard equipment) through a cannula inserted into the left ventricle with a perfusion rate at 7.64 ml/min for young and 15 ml/min for adult animals. After perfusion, brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem. They were then postfixed for 2 h in the same fixative, rinsed in PB and weighed (wet weight). Subsequently, the brains were cryoprotected in sequential solutions of 10, 20 and 30% sucrose in PB.

Brain blocks were serially cut on a freezing microtome (Leitz Wetzlar) into 50 µm-thick sections along the parasagittal plane of each hemi-brain. All sections were collected serially in PB and arranged in six series. The atlas of (Paxinos & Watson, 1986) was used to delimit the cytoarchitetonic brain regions. The series of sections used for immunohistochemistry was first treated with a 0.01 M citrate buffer, at pH 6.0, at 60° C for 4 h. Thereafter, free-floating sections were rinsed in PB three times and incubated with mouse anti-GFAP monoclonal antibody (Sigma-Aldrich, USA) diluted 1:2000 in PB containing 0.3% Triton X-100 (PBX) and 1% normal goat serum for 48 h at 4° C. Sections were then incubated for 1.5 h in secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs, Burlingame, CA) diluted 1:200 in PBX, and processed for immunoperoxidase staining using the avidin-biotin peroxidase complex (Standard ABC kit, Vector Labs). The

binding of antibodies was revealed using diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated glass slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. To control staining specificity, some sections were subjected to an immunohistochemical procedure, omitting the primary antibody.

Quantitative analysis of the number of astrocytes at the mid-level of Substantia nigra

In order to investigate whether an n-3 dietary restriction could induce astrogliosis in the SN, estimates of the number of astrocytes at the mid-level of this nucleus were obtained from six brains per group processed for GFAP immunohistochemistry. The quantitative analysis was performed in three parasagittal sections from the stereotaxic coordinate corresponding approximately from 1.2 mm lateral (plate nº 80) to 2.4 mm lateral (plate nº 82) following the Rat Stereotaxic Atlas (Paxinos and Watson, 1986). These sections were from one of 6 series obtained from the left side of the brain. A microscope (Leica DMLS) coupled to a high level color camera (Samsung SHC-410 NAD) was used to obtain digital images from the immunoreacted brain sections (40 X /0.85 numeric aperture apochromatic objective plus 2 X magnification of the camera). Adjacent sampling windows were obtained from the entire extension of the SN cytoarchitetonic limits (frame size = 0.01 mm^2). An average of 185 and 207 sampling windows per section were analyzed in the SN of young and adult animals respectively. Image J 1.46 (NIH, USA) software was used to count all GFAP positive astrocytes in each section. Using a double blind approach, all the counts were made by three subjects. Differences between groups of each age were

determined by the Student`s T test using SyStat13 for Windows. For this and all other statistical analysis significant differences were considered for $p \leq 0.05$.

Image acquisition and selection for astrocyte complex morphology analysis (fractal analysis)

Considering the heterogeneity of astrocytes and knowing that when these cells became activated they undergo a series of morphological changes, we applied a fractal dimension (FD) analysis as a descriptive parameter for the morphological complexity of these cells in the SN and striatum. Moreover, we also analyzed other morphometric parameters such as the lacunarity and arborization cell area (see below). Digital images were obtained from DAB stained brain slices immunoreacted for GFAP, from 5 animals per group. From one series containing 6 parasagittal brain sections, two or three mid-level sections of the SN or striatum, respectively, were analyzed per animal, corresponding to the stereotaxic coordinate ~1.8 mm lateral to 2.4 mm lateral from (Paxinos & Watson, 1986).

Pictures from two random areas per section of the SN (= 4 pictures/animal) and striatum (= 6 pictures/animal) were taken using an automated upright microscope (Leica, DFC 345 FX camera coupled to a Leica, DM 5500-B microscope, 20x objective). Images from several z-focal planes were taken covering the thickness of the tissue showing GFAP staining in order to capture all the 3D-cell projections in the tissue. Using the ImageJ software (NIH, USA), binary images of the z-stack projection of all individual cells were obtained and their corresponding skeletonized outputs were used for fractal analysis. A minimum of 260 or 360 skeletonized cells per animal were analyzed from SN and striatum, respectively.

Quantitative fractal analysis

Fractal analysis was performed using ImageJ plugin FracLac (Karperien, A., FracLac for ImageJ, version 2.5. http://rsb.info.nih.gov/ij/plugins/fraclac/ LHelp/ Introduction. htm. 1999-2012) using the box counting method. This method systematically covered the image with 6 differently positioned series of decreasing caliber boxes, from 45% of the region of interest (ROI) up to 2 pixels, and registered the number of boxes which contained information from the ROI. Thus, mean values for fractal dimension (Df=log N/log ε, N=count and ε=box size); lacunarity, a measure of heterogeneity and gaps ($\lambda_{\varepsilon,g}=(\sigma/\mu)^2_{\varepsilon,g}$; σ = standard deviation, μ= mean for pixels per box with size ε at grid orientation g) and arborization area (area of the convex Hull, a polygon obtained by connecting the longest astrocytic projections) were obtained from the 6 grid positions used for each skeletonized cell. Differences between groups were determined by the Mann-Whitney Rank sum test using OriginPro 8 Software (Northamptom, MA, USA).

Results

Body and Brain weights

Body weights of young and adult animals were significantly smaller in the DY (71.15 ± 10.94 g; *n* = 39; *P* = 0.0001, unpaired T-test) and DA (339.89 ± 37.30g *n* =21; *P* = 0.0002, unpaired T-test) groups as compared to their respective controls (CY = 81.84 ± 13.40 g; *n* = 32 and CA = 395.84 ± 44.88 g *n* = 16). Significant intergroup difference in brain weights was observed in young (1.48 ± 0.12 g versus 1.69 ± 0.06 g in the control; *n* =7; *P* = 0.015, unpaired T-test) but not in adult animals (1.91 ± 0.12 g versus 2.05 ± 0.18 g, *n* =7). Nevertheless, the brain weight /body weight ratio did not differ between the groups in neither age.

Glutamine synthetase activity in the SN and striatum of young and adult animals

Figure 1 shows the results obtained in the SN and striatum of control and deficient groups. A one-way between groups ANOVA was conducted to compare the effect of n-3 deficient diet on GS activity within each region at young and adult ages and between ages. There was a significant different among the means of the 8 groups at the $P< 0.05$ level [$F(7, 26) = 22.20$, $P = 6.9 \times 10^{-8}$]. *Post hoc* comparisons using the Bonferroni test indicated that in the SN, no intergroup difference in the GS activity was observed between young animals (37.64 ± 5.37 versus 42.86 ± 5.40 gamma-glutamyl hydroxamate/h/mg prot in the CY group). On the other hand, a 2.5 fold increase was observed in the striatum of DY group (58.31±14.71) in comparison to its respective control (CY= 22.41 ± 4.54 gamma-glutamyl hydroxamate/h/mg prot. $P = 5.1 \times 10^{-5}$) and to values obtained in the SN of the same DY group (37.67 ± 4.31 gamma-glutamyl hydroxamate/h/mg prot). At adulthood, GS activity significantly increased in the SN ($P = 7.3 \times 10^{-7}$) and striatum ($P = 0.008$), compared to control. The average values obtained in these regions were respectively 74,65 ± 8,21 and 52,82 ± 8,68 gamma-glutamyl hydroxamate/h/mg prot, which are 4- and 2-fold greater than those of the control groups (18,84 ± 1,01 and 26,11 ± 4,40 gamma-glutamyl hydroxamate/h/mg prot).

Please, insert Figure 1 around here

Number of GFAP positive astrocytes at the mid-level of SN in young and adult animals

Figure 2 depicts low magnification images of representative sampling fields of GFAP immunoreactive parasagittal sections through SN (panel A) and striatum (panel B),

of young and adult control and deficient groups. In the SN, quantitative analysis carried out on 5 animals per group (Fig. 2C) showed a lower average number (~40%) of $GFAP^+$ astrocytes in the DY group (4,777 ± 1,929 cells; $n = 5$) compared to the control (7,716 ± 1,245 cells; $P = 0.04$, 2-tail T-test; $n = 5$). In adult animals, a smaller (~20%) but still significant lower number of $GFAP^+$ astrocytes was detected in the SN of the DA group (7,687.3 ± 1,226 cells; $n = 5$) in comparison to control (9,559 ± 1,337 cells; $P = 0.05$, 2-tail T-test; $n = 5$).

Please, insert Figure 2 around here

Morphometric analysis of GFAP immunoreactive astrocytes in the Substantia Nigra and Striatum

Figure 3 shows skeletonized images obtained from the z-projected pictures used in the analysis of descriptive parameters for astrocyte morphological complexity. Samples of these skeletonized images were obtained from the SN and striatum of young and adult animals. As can be seen, $GFAP^+$ astrocytes in the SN of n-3 deficient groups show diverse forms but their processes are usually longer than that observed in the control condition. In the striatum of the deficient group, usually we can see thin and more ramified processes, when compared to control.

Please, insert Figure 3 around here

Quantification of morphometric analysis of SN and striatum astrocytes through of fractal dimension, lacunarity and arborization area was carried out using two tail Mann Whitney Test and is illustrated in **Figure 4**. In the SN, fractal dimension index did not differ between the groups either at young ($P = 0.754$; CY $n = 260$; DY $n = 317$) or adulthood ($P = 0.067$; CA $n = 351$; DA $n = 331$). On the other hand, increased astrocyte fractal dimension was detected in the striatum of n-3 restricted young ($P = 5.967 \times 10^{-5}$;

CY n = 360; DY n = 378) and adult animals (P = 0.046; CA n = 404; DA n = 378) when compared to their respective controls. Considering the same number of cells per group above described, the lacunarity index was higher in the SN of n-3 restricted young animals ($P = 1.407 \times 10^{-5}$) in comparison to control, but no intergroup difference was detected in the adult groups (P = 0.704). In the striatum this index did not differ between the groups either at young (P = 0.211) or adulthood (P = 0.670). Regarding astrocyte arborization area, a significant increase was detected in the SN of n-3 restricted young animals ($P = 7.313 \times 10^{-5}$) and at adulthood (P = 0.016) compared to controls. Increased arborization area was also induced by the deficient diet in the striatum of young ($P = 1.366 \times 10^{-6}$) and adult (P = 0.0001) animals, when compared to their respective controls.

Please, insert Figure 4 around here

GFAP isoforms expression

Western blot analysis in homogenates of SN, cerebral cortex and striatum of young animals showed a different pattern of GFAP expression in the DY group when compared to the respective control (**Fig. 2A)**. In the control condition, this protein is predominantly expressed with 50 kDa molecular weight, which corresponds to the GFAP phosphorylated isoform. In the DY group, the expression of this phosphorylated isoform was less intensely labeled in all regions analyzed. In this case, a ~42 kDa isoform was more intensely labeled than the phosphorylated one, in both cerebral cortex and SN. In the latter region, two additional GFAP isoforms were also visualized: one with molecular weight between 42 and 50 kDa and another with molecular weight lower than 42 kDa. In the striatum of DY group, the reduced expression of the 50 kDa isoform was also accompanied by an increase in the 42 kDa when compared to control, but both isoforms were similarly expressed within the experimental group.

In the SN of adult deficient animals, three isoforms of GFAP protein were still visualized when compared to the expression profile detected in the control. Nevertheless, the phosphorylated 50 kDa isoform was more intensely labeled when compared to that found in the SN of deficient young animals. On the other hand, in the striatum, no difference between control and deficient adult groups was detected in the GFAP expression pattern: the phosphorylated 50 kDa isoform was the one mainly expressed when compared to the 42 kDa isoform (Fig. 2B).

Please, insert Figure 5 around here

Discussion

Considering previous evidence that an n-3 deficiency induces neurodegeneration, reduces anti-oxidant resource and BDNF expression in the nigrostriatal system, the present study investigated whether such effects would involve an impairment in astrocyte development or reactivity in the SN. In addition, we also hypothesized that a differential astrocytic reaction could occurs in the striatum of young animals as a potential mechanism involved in its apparent resilience under condition of 50% DHA depletion (Cardoso *et al.*, 2012). The results corroborated our hypothesis that differential astrocyte reactivity can occur in the nigrostriatal system.

Effects on Glutamine Synthetase Activity

Under physiological conditions, GS is mainly expressed in astrocytes and its activity is usually stimulated by elevated glutamate concentrations in the extracellular medium (Zou *et al.*, 2010) but can be also triggered under deleterious conditions when glutamate uptake by astrocytes is reduced (Feoli *et al.*, 2006). The distribution of GS in several regions of the rat central nervous system was described by (Norenberg, 1979) using an immunohistochemical approach. According to Norenberg (1979), the intensity of astrocyte

GS staining varied greatly in different regions, including basal ganglia, and correlated well with sites of higher glutamatergic activity. Modifications in the mRNA expression of GS were recently demonstrated in the hippocampal CA1 area as a consequence of n-3 deficiency or its supplementation in the diet (Harbeby *et al.*, 2012). As stated by this study both conditions were able to increase the mRNA level of this enzyme as well as the glutamate transporter GLAST. Our results with young animals fed an n-3 deficient diet, indicated that a higher GS activity in the astrocytes of the striatum occurred simultaneously with a greater tSOD activity in this nucleus, as reported by (Cardoso *et al.*, 2012) , using the same experimental model as we did. Considering that the glutamatergic activity in the striatum is high compared to the SN, an increased GS activity in this nucleus could favor the activity-dependent regulation of energy metabolism by astrocytes. In addition, could reduce glutamate excitotoxicity, usually associated to increased levels of oxidative stress (Kim *et al.*, 2000; Nguyen *et al.*, 2011). Taken together, these results reinforce our hypothesis that in the striatum of n-3 deficient young animals, a differential enzymatic reaction involving astrocytes contributed to the resilience of this nucleus to oxidative insult.

In the SN, the lack of intergroup difference in the GS activity observed in young animals contrasts with the expressive increase in this activity (~4 fold) in n-3 restricted adult animals, even when the number of $GFAP^+$ astrocytes is 20% lower than that found in the control condition. It should be noted that in the SN of these adult animals, the loss of dopaminergic neurons increased to 40% concomitant with higher levels of oxidative stress when compared either to young animals or to respective adult control. Moreover, fewer number of $BDNF^+$ cells in the SN and higher levels of nitric oxide in the striatum were also detected in n-3 deficient adult animals (Cardoso *et al.*, 2013).

At least two aspects are of interest in the discussion of increased GS activity as a function of age in the SN. First, it is tempting to propose that could be a consequence of a

delay in astrocyte development induced by DHA deficiency. Using a multigenerational model of linolenic acid deficiency, Bourre *et al.*, (1984) reported that DHA depletion in astrocyte phospholipids was higher at 15 days old (6 fold) than at adulthood (3 fold), whereas the opposite effect was observed in neurons. Therefore, it is possible that an increased DHA incorporation in astrocyte membranes occurred with age, in spite of the progressive DHA loss in the whole tissue. Such incorporation favored their reactivity and minimized negative effects on their proliferation as will be discussed below. Second, a number of studies have reported that some conditions of neurodegeneration induce GS expression and activity by neurons (Fernandes *et al.*, 2010) or microglia especially when astrocyte function is impaired (Chretien *et al.*, 2002; Gras *et al.*, 2006). Preliminary data from our research has detected phenotypic changes in the SN microglia, suggestive of a reactive condition, in the n-3 restricted group at adulthood, (data not shown) and further experiments are in course in order to address this issue. Thus, at this moment, we cannot also discard the possibility that other types of cells, in addition to astrocytes, can be contributing to the GS activity in the SN of n-3 deficient adult animals.

Effects on astrocyte number and phenotypic changes

It is well established that after different types of injury to the brain, astrocytes can undergo various phenotypic changes that enable them to both respond to and play a role in pathological processes (Eddleston & Mucke, 1993; Verkhratsky *et al.*, 2013). In the present study we observed that an increased astrocyte arborization area occurred in the SN of both young and adult n-3 restricted animals. These morphological changes were distinct from those found in the striatum. In this latter nucleus, increased index of complexity indicated by fractal dimension in addition to higher arborization area occurred in young

and adult animals. It is noteworthy that such effects in the striatum of young animals occurred in the absence of oxidative stress in this nucleus (Cardoso *et al.*, 2012).

The modifications observed in the astrocyte arborization area in the SN of animals fed the experimental diet, occurred concomitant with a lower number of $GFAP^+$ astrocytes in comparison to control condition. Nevertheless, while in young animals the average number of $GFAP^+$ cells in the SN was ~40% smaller than that detected in the respective control, at adulthood the significant intergroup difference was reduced to ~20%. A number of studies have shown that consistent results in glial reactivity can be obtained when data of fractal dimension is complemented by measures of lacunarity, which indicates change in the image density, gaps or spaces between processes (Karperien *et al.*, 2013). The higher lacunarity index found in the SN of young animals fed the experimental diet is consistent with the lowest number of astrocytes detected in this nucleus when compared to the respective control or to the deficient animals at adulthood.

Under physiological conditions, SN is recognized as a nucleus where the number of glial cells surrounding dopaminergic neurons is the lowest, compared to other brain areas, and this fact contributes to increase the vulnerability of these neurons to lesions (Mena *et al.*, 2002). Although our present findings indicate that under conditions of DHA deficiency, at least one type of astroglial morphological plasticity (increased arborization area) can occur in the SN, it is plausible that the harmful effect induced by this nutritional insult on astrocyte proliferation contributed to worsen dopamine cell degeneration previously reported (Passos *et al.*, 2012; Cardoso *et al.*, 2013). These results suggest a higher sensibility of SN astrocytes to n-3 deficiency compared not only to striatum but to other brain regions. In the hippocampal CA1 region, for example, a recent study did not find modifications in the number of $GFAP^+$ astrocytes nor in the expression of GFAP

phosphorylated isoform in adult animals (6 months old) submitted to n-3 restricted diet since conception (Latour *et al.*, 2013).

Effects on GFAP isoforms expression

The presence of astrogliosis in the SN as a consequence of dopaminergic cell loss is a matter of discussion in the literature. Although some experimental models of Parkinson's disease (PD) demonstrated that this can occur, other studies suggest that this type of reaction depends on the time course of cell degeneration or the type of the mitochondrial toxin used (Teismann & Schulz, 2004; McGeer & McGeer, 2008). In studies with humans, for example, there is one report of absence of astrogliosis in the SN of PD (Mirza *et al.*, 2000), while an expressive astrocyte reaction occurred in the striatum (Verkhratsky *et al.*, 2013).

The relative expression of different GFAP protein isoforms is another parameter used in the present study to investigate potential modifications in astrocyte proliferation induced by DHA depletion. It has been well established that GFAP is involved in cellular remodeling during the mitotic, pathological and plastic processes (Rodnight *et al.*, 1997). Among the factors related to these functions, GFAP phosphorylation contributes to the disassembly and consequent depolymerization of this intermediate filament. In addition, this phosphorylation will increase its soluble form, protecting this molecule from proteolytic degradation (Yasui *et al.*, 1998; Takuma *et al.*, 2004). It has also been shown that GFAP phosphorylation is associated with mitotic division during cell proliferation, modulating the cytokinesis (Inagaki *et al.*, 1994; Tsujimura *et al.*, 1994; Sekimata *et al.*, 1996). In some pathological conditions, such as hypoxic-ischemic insult, an increased expression of phosphorilated GFAP has been associated with neuroprotection in the central nervous system (Sullivan *et al.*, 2012). Conversely, GFAP dephosphorylation causes

polymerization and can modify cellular responses (Sullivan *et al.*, 2012). One of the possible mechanisms involved in the GFAP phosphorylation is the increase in the intracellular cyclic AMP, (Pollenz & McCarthy, 1986) which is also related to astrocytic morphological differentiation (Pollenz & McCarthy, 1986; Joardar *et al.*, 2006).

It has been demonstrated in cerebral cortex astrocyte cultures that DHA supplementation in the medium increases the activity of the cyclic AMP dependent protein kinase A. This increased activity induces phosphorylation of several cellular substrates, including the GFAP molecule (Joardar *et al.*, 2006). In line with this effect, Joardar & Das, (2007), using coconut oil as the fatty acid source, reported that DHA deficiency in the astrocyte culture medium modified the expression of GFAP isoforms, when compared to conditions where soya, mustard or linseed oil were used. These authors reported that DHA deficiency increased the expression of neutral and basic non-phosphorylated GFAP isoforms and that such condition impaired astrocyte morphological differentiation, keeping them in an immature phenotype. On the other hand, when treatment of astrocyte cultures was conducted with n-3 fatty acid enriched oils, many of the neutral isoforms of GFAP were not expressed, whereas new acidic variants were detected.

In the present *in vivo* study, we have shown that DHA deficiency induced by a diet containing coconut oil, as the only source of lipids, was also able to reduce the relative expression of 50 kDa GFAP phosphorylated isoform in homogenates of cerebral cortex, compared to the control condition. Similar changes were observed in the striatum and SN of young animals. In the SN, three or two additional GFAP isoforms with molecular weights lower than 50 kDa were visualized either in young or adult animals fed the experimental diet. In contrast, in the striatum of adult animals, intergroup difference was no longer observed. Such results lead us to speculate that the lower expression of the phosphorylated GFAP isoform and lower number of $GFAP^+$ astrocytes in the SN of n-3

restricted groups can be interrelated processes involved in long lasting modifications on astrocyte proliferation in this nucleus. It has recently demonstrated that DHA deficiency in the maternal diet can impair the expression of several genes involved in proliferation and differentiation of rat neural stem cells (Goustard-Langelier *et al.*, 2013). Therefore, further *in vivo* and *in vitro* studies are needed to analyze molecular mechanisms involved in the apparent impaired astrocyte proliferation observed in the SN.

In conclusion, the present findings demonstrate for the first time that essential fatty acid dietary restriction over two generations is able to induce adverse effects on astrocyte morpho-functional parameters in two nuclei of basal ganglia. The results show that the functional impact of DHA deficiency can be region specific and that a delayed astrocyte phenotypic plasticity and proliferation occurs in the SN. Taken together, the present data reinforce the hypothesis that astrocytic dysfunction may contribute to worsen midbrain dopamine cell degeneration induced by an n-3 dietary restriction during brain development. Figure 6 depicts as schematic drawing the main results and hypothesis of the present study.

Please, insert Figure 6 around here

Conflict of interest

There is no conflict of interest in the work reported in the present paper.

Acknowledgements

The authors are grateful to Zenira Cosme Xavier for technical assistance. The acquisition of the reagents used in this work was supported by the Brazilian National Research Council (CNPq); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Procad no. 131/2007 and PROCAD NF-2009), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco - FACEPE (APQ 0036-2.07/11) and Instituto Nacional de Neurociência Translacional (INCT no. 573604/2008-8) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado Rio de janeiro (FAPERJ). We are also

grateful to FACEPE which provided scholarships for David Filipe de Santana, Henriqueta Dias Cardoso and Catarina Gonçalves-Pimentel (DCR 0079-2.07/10). The English version of this text has been revised by Sidney Pratt, BA, MAT (The Johns Hopkins University), RSAdip (TEFL).

Abbreviations

BDNF , brain derived neurotrophic factor; DHA, docosahexaenoic acid; GFAP, glial fibrilary acidic protein; GS, glutamine synthetase; SN, substantia nigra; PD, Parkinson's disease; tSOD, total superoxide dismutase; PUFAs, polyunsaturated fatty acids.

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Figure Legends

Figure 1. Effects on Glutamine synthetase activity

Glutamine synthetase activity in the substantia nigra (SN) and striatum of young **(A)** and adult **(B)** rats fed control (CY and CA, white bars) and n-3 fatty acid deficient (DY and DA, dark grey bars) diets. Each bar represents the mean ± SD obtained from three independent experiments carried out in triplicates. No significant intergroup difference in the glutamine synthetase activity was found in the SN of young animals but an increase of ~2.5 fold was detected in the striatum of DY group ($F_{7, 25} = 22.20$ **$P = 5.1 \times 10^{-5}$ vs respective CY and *$P = 0.046$ vs SN of CY. At adulthood, SN: DA vs CA $^{###}$ $P = 7.3 \times 10^{-7}$; striatum: DA vs CA $^{##}$ $P = 0.008$; SN DA vs striatum DA $^{#}$ $P = 0.05$.

Figure 2. Astrocyte number and phenotypic changes in the substantia nigra and striatum

Low magnification images of representative sampling fields of GFAP immunoreactive parasagittal sections through SN (**panel A**) and striatum (**panel B**). Note the lower density of astrocytes and their longer processes in the SN of young (DY) and adult (DA) animals fed with n-3 deficient diet, in comparison to control (CY and CA). **C:** Average number of GFAP immunoreactive astrocytes in three parasagittal sections at the mid-level of substantia nigra in young and adult rats ($n = 5$ per group) fed control (white bars) and n-3 fatty acid deficient (grey bars) diets. Bars represent mean ± standard deviation. *Indicates a significant difference between groups of young ($P = 0.042$) and adult ($P = 0.050$) animals according to Unpaired two tail Student`s T test.

Figure 3. Representative skeletonized cells used for fractal analysis.

Representative skeletonized cells obtained from z-stack projections of $GFAP^{+}$ astrocytes of substantia nigra (SN) and striatum of young (CY and DY) and adult animals (CA and DA) of control and deficient groups. Such projections were used for boxes reporting the fractal analysis count. C = control group; D = n-3 fatty acid deficient group; Y = young; A = adult.

Figure 4. Quantitative analysis of Fractal dimension, lacunarity and arbor area of astrocytes.

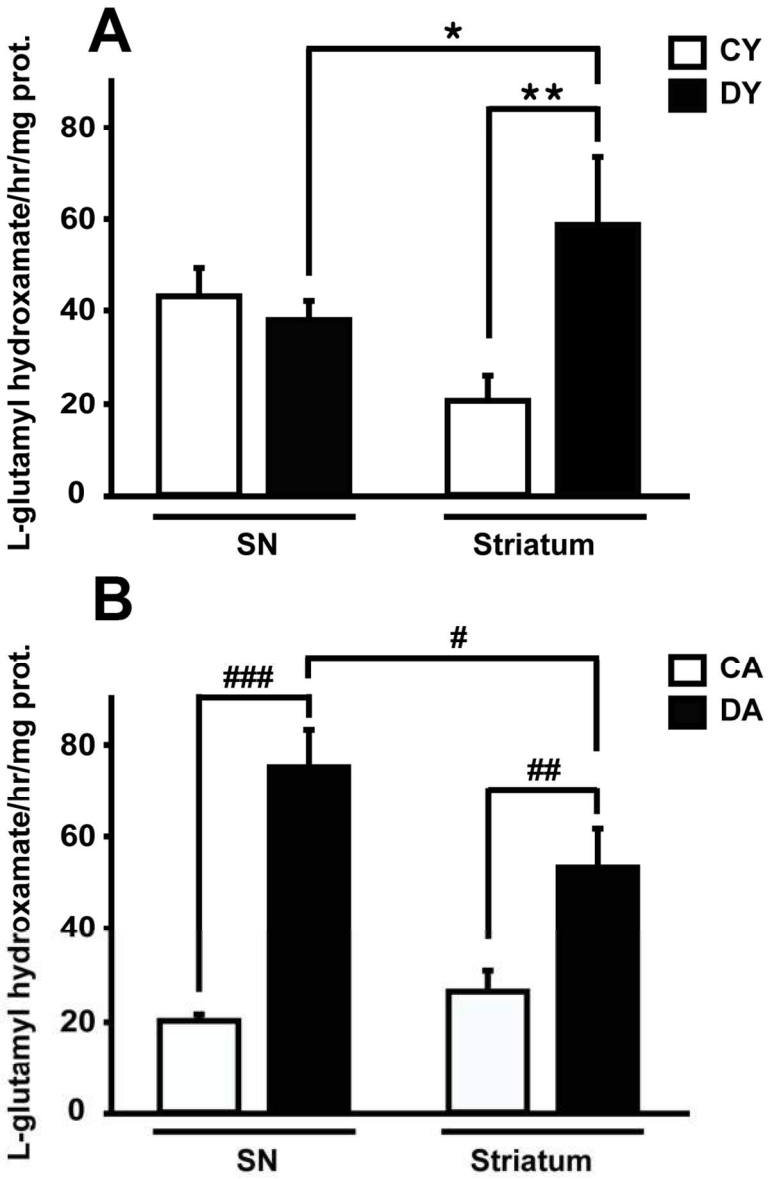
Box plots show mean distribution values (see fractal analysis methods) of fractal dimension, lacunarity and arbor area of astrocytes from young and adult rats fed control and essential fatty acid deficient diets. On the box plot, the line indicates the median, the box represents the 25^{th} and 75^{th} percentile, and bars show minimum and maximum values. Circles on the plot area represent outliers. * $P = 0.046$; ** $P = 5.967 \times 10^{-5}$; $^{###}$ $P = 1.407 \times 10^{-5}$; $^{\$\$\$}$ $P = 7.313 \times 10^{-5}$; $^{\$\$}$ $P = 0.016$; §§§ $P = 1.355 \times 10^{-6}$; §§ $P = 0.0001$, compared to respective control groups (Two-tailed Mann-Whitney Rank sum test). C = control group; D = deficient group.

Figure 5. Expression profile of GFAP protein isoforms

Expression profile of GFAP protein isoforms in the striatum, cerebral cortex and substantia nigra of young animals **(A)** and in the striatum and substantia nigra of adult animals **(B)** fed control (C) and n-3 fatty acid deficient (D) diets. Note the reduction in GFAP-immunoreactive band of 50 kDa in all the regions analyzed and the greater expression of ~42 kDa isoform in cerebral cortex and substantia nigra (SN) of deficient animals compared to control. In the substantia nigra, two additional bands were also detected in the

deficient group at both ages. Molecular weight marker and purified GFAP from bovine brain were used as control (GFAP lane).

Figure 6. Schematic drawing illustrating the main results obtained in the present and in previous studies using the same experimental model to analyze the impact of n-3 deficiency in the nigrostriatal system. The apparent resilience of astrocytes in the striatum contrasts with the potential contribution of SN astrocyte vulnerability to neurodegeneration induced by DHA depletion in this nucleus.



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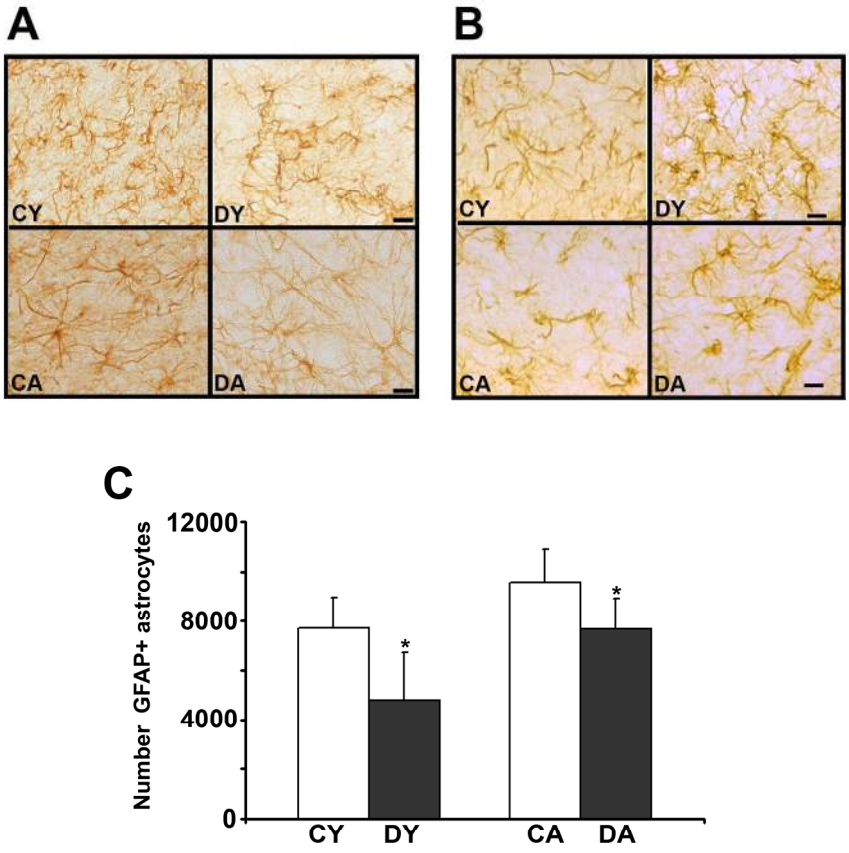

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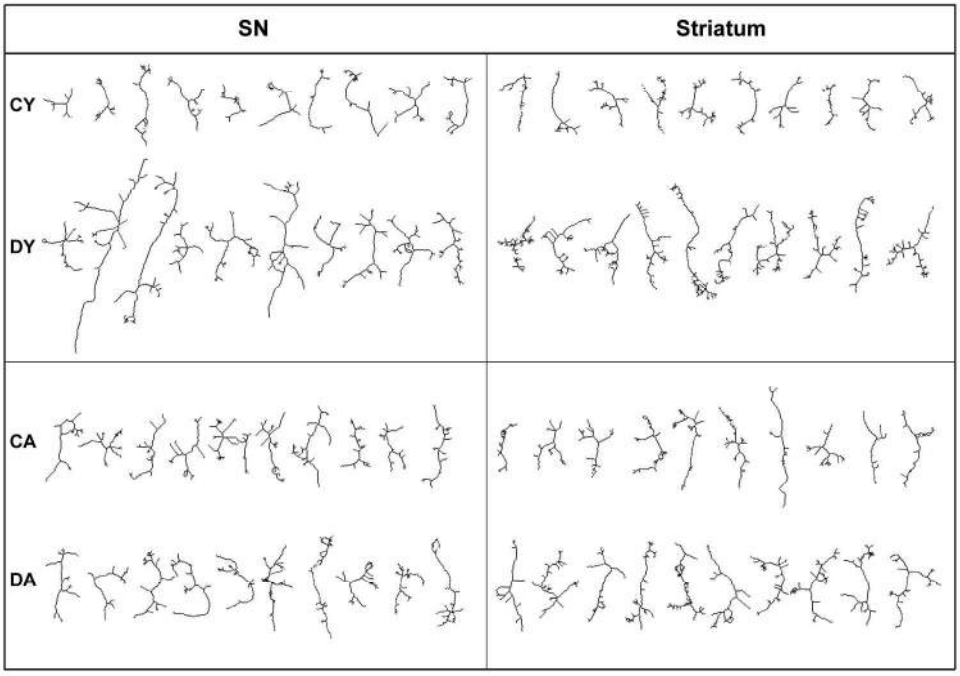

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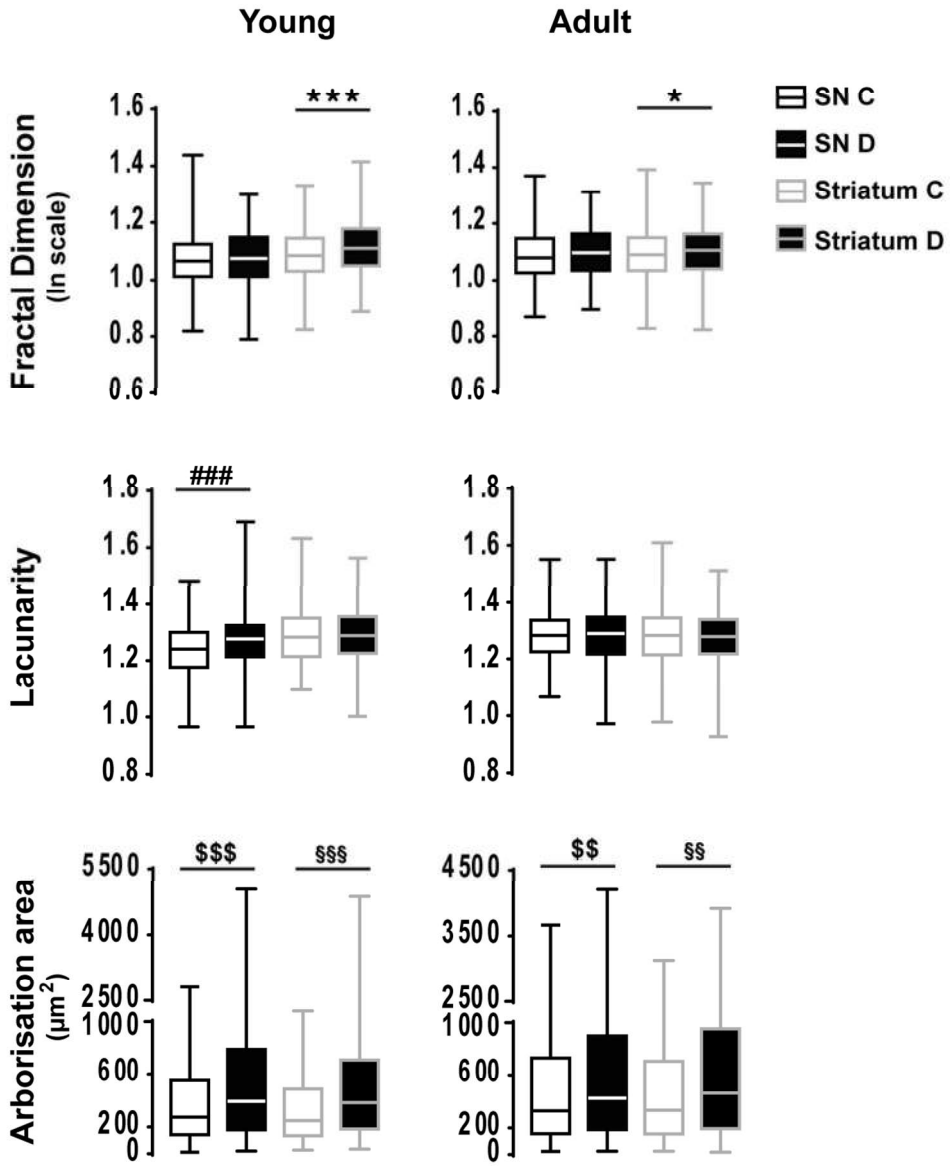

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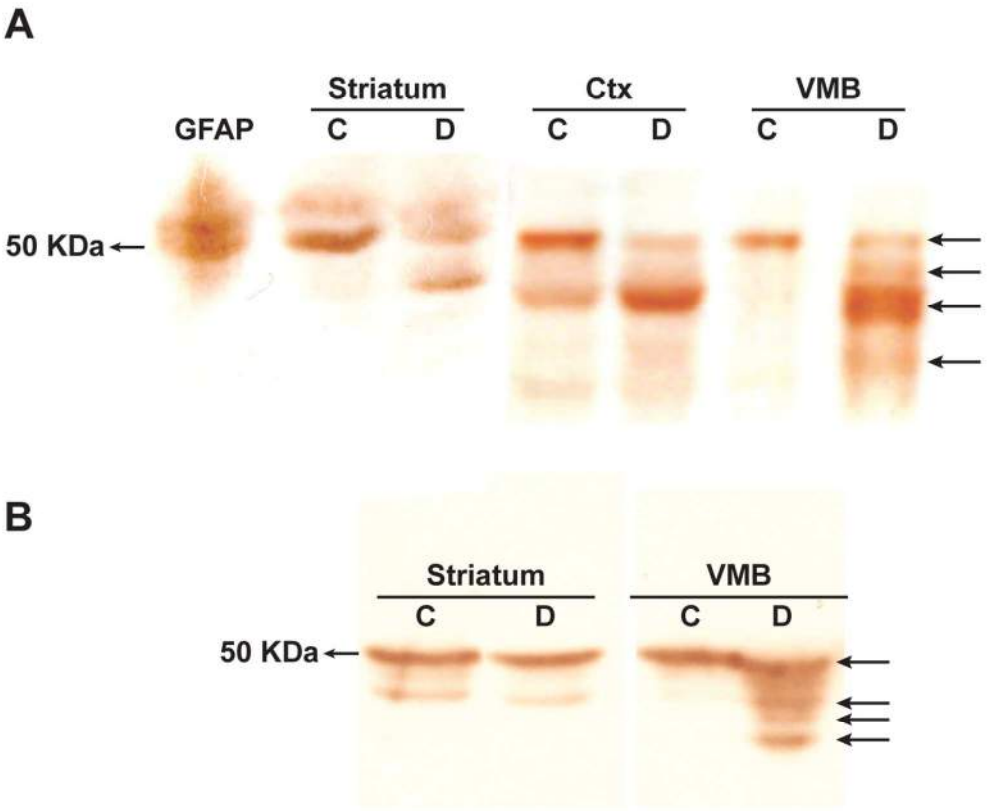

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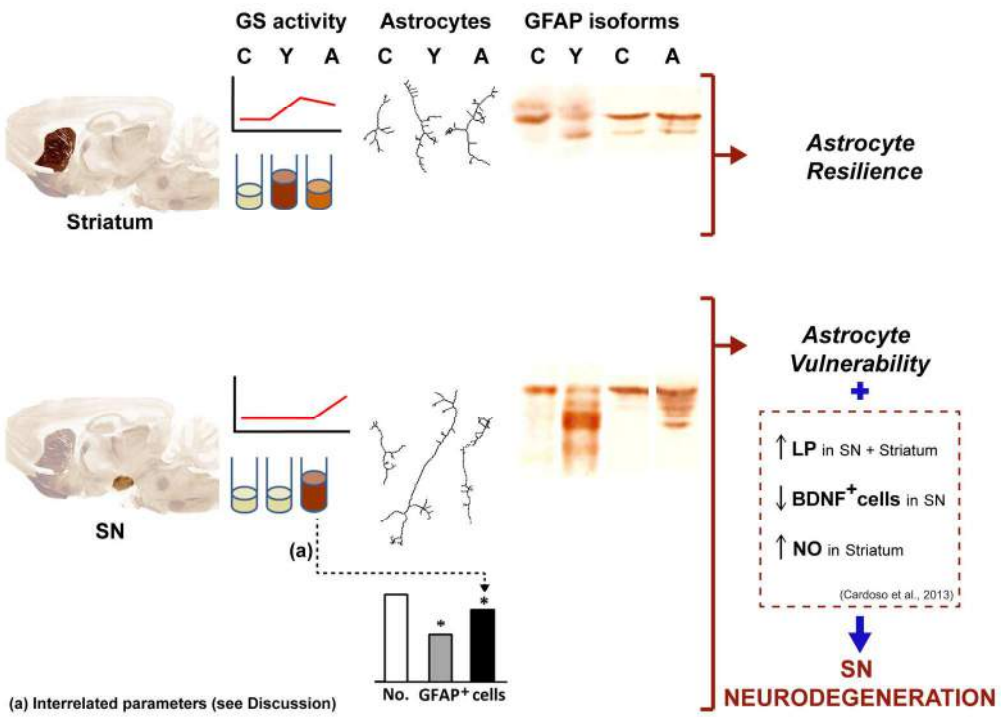

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Table 1. Diet Composition (grams/100g diet)

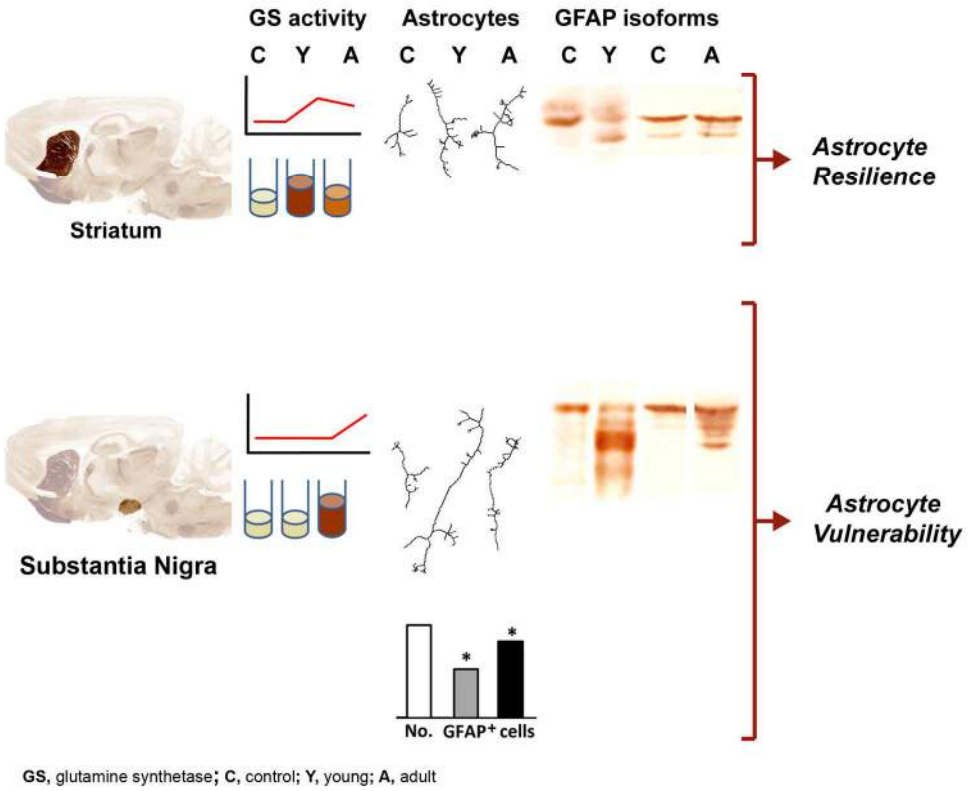
Ingredients	Control diet	Experimental diet
Casein	20.7	20.7
Cellulose	1.8	1.8
Corn starch	46.8	46.8
Sucrose	21.0	21.0
Soyabean oil	5.0	-
Coconut oil	-	5.0
Vitamin mix [1]	0.9	0.9
Mineral mix [2]	3.7	3.7
D.L-Cystine	0.1	0.1
Butyl hydroxytoluene	0.001	0.001
Kcal/100g	399.1	400.5

[1] **Vitamin mixture** (Rhoster Ind.Com. LTDA. SP. Brazil) containing (m%): folic acid (20); niacin (300); biotin (2); calcium pantothenate 160; pyridoxine (70); riboflavin (60); thiamine chloride (60); vitamin B_{12} (0.25); vitamin K_1 (7.5). Additionally containing (UI%): vitamin A 40.000; vitamin D_3 10.000; vitamin E (750). [2] **Mineral mixture** (Rhoster Ind. Com. LTDA. SP. Brazil) containing (m%): $CaHP0_4$ (38); K_2HP0_4 (24); $CaCO_3$ (18.1); NaF (0.1); NaCl(7.0); MgO (2.0); $MgS0_4$ $7H_20$ (9.0); $FeS0_4$ $7H_20$ (0.7); $ZnS0_4$ H_20 (0.5); $MnSO^+$ H_20 (0.5); $CuS0_4$ $5H_20$ (0.1); Al_2 $(S0_4)_3K_2S0_4$ $24H_20$ (0.02); Na_2SeO_3 $5H_20$ (0.001); KCl (0.008).

Table 2. Fatty acid composition of the diets
(% of total fatty acids)

Fatty acids	**Control diet**	**Deficient diet**
8	0.02	3.27
10	0.03	3.95
11	nd	0.07
12	0.20	28.04
13	nd	0.06
14	0.19	19.56
15	0.02	0.02
16	9.27	11.32
17	nd	0.02
18	15.31	0.72
20	0.33	0.16
22	0.51	0.08
23	0.07	0.02
24	0.04	nd
Total saturated	**26.01**	**67.29**
16:1	2.72	0.06
18:1n9	9.36	23.51
20:1	0.24	0.16
Total monounsaturated	**12.32**	**23.73**
18:2n6	55.36	8.10
18:3n3	6.04	0.49
20:2	0.04	0.06
20:5n3	0.03	nd
22:2n	0.05	0.04
22:6n3	0.13	0.06
Total polyunsaturated	**61.65**	**8.75**
18:2n6 /18:3n3	9.17	16.39

nd = not detected



GS, glutamine synthetase; C, control; Y, young; A, adult

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Abstract gráfico

Omega-3 fatty acid deficiency induces oxidative stress and neurodegeneration in the nigrostriatal system.To understand whether astrocyte dysfunction occurs in this condition, we assessed glutamine synthetase activity, morphological changes, signs of proliferation and GFAP expression. The results show that the impact of DHA deficiency is region specific within the basal ganglia. A delayed astrocyte reactivity and proliferation in the SN may contribute to dopamine cell degeneration in this nucleus

APÊNDICES

APÊNDICE D - Capítulo de livro submetido para publicação pela "Rede Glial Luso-Brasileira" através da Universidade de Coimbra, Portugal: Restrição dietética em ácidos graxos ômega-3 induz reatividade fenotípica na microglia da substantia nigra de ratos

RESTRIÇÃO DIETÉTICA EM ÁCIDOS GRAXOS ÔMEGA-3 INDUZ REATIVIDADE FENOTÍPICA NA MICROGLIA DA *SUBSTANTIA NIGRA* DE RATOS

TITLE: Nutritional restriction of omega-3 fatty acids induces phenotypic plasticity in the microglia of rat *substantia nigra*

Autores: [1]Isaac, A.R. [1]Gonçalves-Pimentel, C. [1]Silva, E.A.N. [1]Santos-Junior, E.F. [1]Moreno, G.M.M. [1]Seabra, M.A.B.L. [1]Santos, R.V.C. [1]Augusto, R.L. [2]Guedes, R.C.A. e [1]Andrade-da-Costa, B.L.S.*

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Resumo

Deficiência crônica em ácido docosahexaenóico (DHA) induz neurodegeneração no sistema nigro-estriatal de ratos. Para investigar se atividade microglial contribui para este efeito, o presente estudo analisou alterações fenotípicas indicativas de seu estado reativo na *substantia nigra*. Ratos foram mantidos a partir da concepção em dietas contendo níveis adequados ou deficientes em ácido alfa-linolenico e analisados na idade adulta. Os resultados evidenciaram alterações morfológicas e marcação para a lectina obtida do Lycopersicum esculentum na microglia do grupo deficiente em DHA em comparação ao controle. Os achados sugerem que reatividade microglial pode contribuir para a neurodegeneração induzida pela deficiência em DHA na *substantia nigra*.

Abstract

Chronic docosahexanoic acid (DHA) deficiency induces neurodegeneration in the rat nigrostriatal system. To investigate whether microglia activity in the *substantia nigra* contributes to this effect, this study analyzed phenotypic changes indicative of their reactive state. Rats were raised from conception in diets containing adequate or deficient levels of alpha-linolenic acid and analyzed in adulthood. The results showed morphological changes and labeling with lectin obtained

from *Lycopersicum esculentum* in the microglia of deficient group in comparison to the control. The findings suggest that microglial reactivity can contribute to *substantia nigra* neurodegeneration induced by DHA deficiency

INTRODUÇÃO

O ácido graxo polinsaturado docosahexaenoico (DHA) da família ômega-3 e seus metabólitos desempenham funções essenciais durante o desenvolvimento do sistema nervoso (Innis, 2007). Além de constituinte estrutural das membranas, atua como sinalizador de eventos moleculares capazes de modular mecanismos epigenéticos (Goustard-Langelier *et al*., 2013), interações intra e inter-celulares e a transcrição gênica (Yavin, 2006). Níveis balanceados de DHA e do ácido araquidônico (AA) da família ômega-6, são necessários ao adequado metabolismo cerebral, em parte pelas ações opostas que estes podem exercer sobre mecanismos inflamatórios (Schimidtz *et al.,* 2008) envolvidos em algumas doenças degenerativas (Bousquet *et al.*, 2011). Na doença de Parkinson, por exemplo, a neurodegeneração da substantia nigra (SN) tem sido associada, dentre outros fatores, ao estado inflamatório decorrente da reatividade microglial (Croisier et al., 2005; Sanchez-Guajardo et al., 2013). Neste contexto a suplementação dietética do DHA tem sido indicada como uma alternativa nutracêutica por aumentar a síntese de eicosanóides anti-inflamatórios (Calder, 2012) e por exercer ações inibitórias sobre a expressão de fatores de transcrição gênica envolvidos na síntese de citocinas pro-inflamatórias (Ji *et al*, 2012).

Evidências recentes indicam que desequilíbrio nos níveis de ômega-3 e 6 na dieta materna induzem reatividade microglial no estriado (Kuperstein *et al.,* 2008; razão ω6/ω3=173) e alteram fenótipo e motilidade da microglia no hipocampo, durante o aleitamento(Madore *et al.,* 2014; razão ω6/ω3=280). Tais resultados, no entanto, não refletem apenas o efeito de uma deficiência de DHA uma vez que níveis de AA foram aumentados. Utilizando um modelo de restrição dietética crônica em ácidos graxos essenciais, capaz de induzir depleção de DHA (~65%) sem afetar o AA encefálico, evidenciamos neurodegeneração na *substantia nigra* (SN) associada a elevados níveis de óxido nítrico e lipoperoxidação, além de redução na expressão de BDNF em animais adultos (Cardoso et al., 2012, 2013).

Neste estudo, considerando as evidências de modulação do DHA na sinalização neuroinflamatória, hipotetizamos que a deficiência em DHA per se, na ausência de elevados níveis de AA induz reatividade

fenotípica na microglia da SN como um dos mecanismos envolvidos na neurodegeneração observada neste núcleo (Cardoso et al., 2013).

MÉTODOS UTILIZADOS

Ratos Wistar foram subdivididos em dois grupos de acordo com as dietas, as quais diferiram quanto à composição adequada (controle) ou reduzida (deficiente) em ácidos linoleico e alfa-linolênico (Cardoso et al., 2013). Tal dieta foi mantida por duas gerações e os encéfalos de animais machos (90-100 dias) da segunda geração foram analisados. Após perfusão transcardíaca dos animais, secções parassagitais (45 µm) dos encéfalos foram obtidas em criostato (Leica). Para visualização da microglia foram utilizados o anticorpo monoclonal anti-IBA-1 feito em coelho (Wako Chemical) e a lectina do tomate *Lycopersicum esculentum (*Sigma-Aldrich*)* sendo a imunomarcação obtida pelo complexo avidina-biotina-peroxidase (Vector Labs) e o cromógeno diaminobenzidina. Análise do fenótipo morfológico da microglia foi realizada em 3 animais por grupo, nos limites citoarquitetônicos da SN (nível medial). Três secções parassagitais por animal, foram analisadas. Imagens digitalizadas foram obtidas (microscópio Nikon DS-Fi1; objetiva 100x/1.25) e o programa Image J, 1.45s, NIH, USA utilizado para análise morfométrica .

RESULTADOS

O painel apresentado na figura 1 ilustra imagens de regiões da SN de 2 animais representativos dos grupos controle ou deficiente em ômega-3. Em ambos os grupos as células da microglia imunorreativas à proteína IBA-1, encontram-se uniformemente distribuídas ao longo deste núcleo . Porém, é possível detectar que no grupo deficiente várias células da microglia apresentam uma imunorreatividade mais intensa caracterizada pelo espessamento do corpo celular e prolongamentos, (Fig 1C e 1D) quando comparadas às observadas no grupo controle (fig 1A e 1B). Análise quantitativa de parâmetros morfométricos realizada ao nível medial da SN evidenciou um aumento significativo ($p< 0.0001$; Mann Whitney test) na área do soma (mediana 62.12 μm^2; min=24.16; max:125.33) e na forma elipsoidal dada pela diferença entre o diâmetro máximo e mínimo (mediana 5.281 µm; min=1.03; Max=8.51) da microglia do grupo deficiente (n=318) comparado ao grupo controle (medianas 44.27 μm^2 [17.63-86.50 μm^2] e 3.92 µm [1.06-8.70 µm] n= 300).

Além das alterações morfológicas observadas nas células do grupo deficiente foi também detectada imunomarcação para a lectina do tomate *lycopersicum esculentum*, que detecta microglia em diferentes estágios de reatividade e neuroinflamação (Pelaez et al., 1999; Borner et. Al., 2011). As figuras 1E e 1F ilustram respectivamente, imagens representativas da SN de animais dos dois grupos, evidenciando marcação da lectina em vasos, no grupo controle e em vasos e células da microglia no grupo deficiente em DHA.

DISCUSSÃO E CONCLUSÕES GERAIS

Modificações na forma e dimensão do corpo celular da microglia tem sido correlacionadas com os diferentes estágios de atividade que a mesma apresenta em condições de neuroinflamação (Torres-Platas et al 2014). Neste estudo evidenciamos que uma deficiência específica em DHA, sem elevação dos níveis de AA, é capaz de promover alterações fenotípicas na microglia da SN indicativas de um estado reativo. Os resultados reforçam a idéia de um efeito modulatório do DHA sobre a função da microglia (Ji et al 2012; Pettit et al 2013) e enfatizam a importância do aporte adequado deste ácido graxo para minimizar condições neurodegenerativas.

***Uma a Três Conclusões em bullet point**

- Deficiência em ômega-3 aumenta a vulnerabilidade da substantia nigra à neuroinflamação

- Deficiência em ômega-3 per se induz reatividade microglial na substantia nigra

Agradecimentos

Este trabalho teve apoio financeiro fornecido pelo CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Procad no. 131/2007 and PROCAD NF-2009), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco - FACEPE (APQ 0036-2.07/11), Instituto Nacional de Neurociência Translacional (INCT no. 573604/2008-8).

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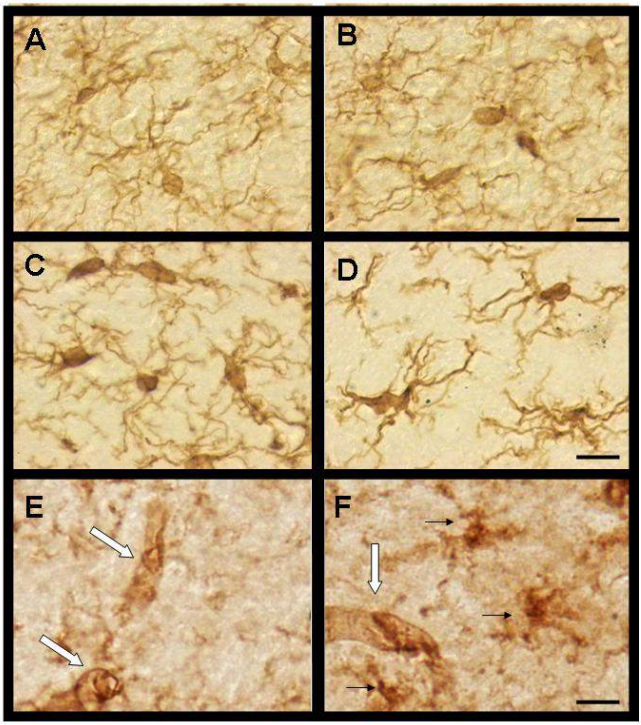
Legenda da Figura

Figura 1: Alterações fenotípicas induzidas pela deficiência em DHA na microglia da substantia nigra, evidenciada pela imunorreatividade à proteína IBA-1(A e B no grupo controle) (C e D no grupo deficiente) e à lectina do tomate *Lycopersicum esculentum (E,* controle e F, deficiente*)*. Observar alterações na dimensão e forma do corpo e prolongamentos celulares. Setas brancas em E e F indicam marcação de vasos e setas pretas marcação de microglia reativa. Escala = 10 μm.


A
B
C
D
E
F

APÊNDICES

APÊNDICE E - Resumo de artigo ser submetido (colaboração): In vivo and in vitro studies of thiol - capped CdTe aqueous colloidal quantum dots as a tool for brain tumor diagnostics

IN VIVO AND *IN VITRO* STUDIES OF THIOL - CAPPED CdTe QUANTUM DOTS AS A TOOL FOR BRAIN TUMOR DIAGNOSTICS

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ABSTRACT

Glioblastoma (grade IV) is the most aggressive and infiltrating tumor of the central nervous system (CNS), showing a variety of mutations as well as high degree of vascularity, cell polymorphism and nuclear atypia. Unfortunately early diagnostic of brain tumors is hard, as imaging tools are not efficient for proper diagnosis of these types of tumors, leading to treatment failures. Here we describe a new in vivo targeting and imaging method for U87 glioblastoma tumor type xenotransplanted into male swiss mice brain using aqueous colloidal CdTe quantum dots (CdTe QDs) conjugated to anti-glial fibrillar acidic protein (GFAP). We have synthesized and optimized anti-GFAP conjugated red-emitting CdTe QDs to label also U87 tumor cell line *in vitro* and tested their ability to be incorporated in healthy cerebral cortex astrocyte primary cultures. The toxicity of isolated green (530 nm) or red (644 nm) emitting CdTeQDs synthesized for 2 or 10 h was evaluated using MTT assay applied to U87 cells. The tumor growth was visualized inside the brain by the hematoxylin and eosin staining and showed the successful delivery of the U87 cells into the brain parenchyma. CdTe QDs conjugated to anti-GFAP were injected into the tumor region and their uptake by the U87 cell line was visualized by fluorescence microscopy, showing a very specific double-labeling of vimentin-immunoreactive glioblastoma. Compared to U87 tumor cells that easily taken up anti-GFAP conjugated red-emitting CdTe QDs, healthy astrocytes kept, in primary cultures, offered more resistance to their incorporation and were weakly labeled. The results reported here provide new perspectives for using CDTe QDs in gliobastoma detection, suggesting their potential application in imaging-guided surgery.

Keywords

Quantum Dots, U87 cell line, Glioblastoma, Xenograft model, Fluorescence microscopy

APÊNDICES

ANEXO A - Parecer do comitê de ética em experimentação animal da UFPE

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Recife, 23 de julho de 2013.

Ofício nº 566/13

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Profª Belmira Lara da Silveira Andrade Costa**
Universidade Federal de Pernambuco
Departamento de Fisiologia e Farmacologia
Processo nº 23076.055875/2012-11

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"Repercussões de Desnutrição Sobre o Sistema Nervoso Entérico – Caracterização da Glia Entérica do Intestino Grosso ".**

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

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

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

Origem dos animais: Biotério do Departamento de Fisiologia e Farmacologia/UFPE. Animais: ratos; Linhagem: Wistar; Idade: progenitores adultos e prole jovem (30-40 dias) e adulta (90-110 dias); Peso: jovens (50-80g) e adultos (280-380g); Sexo: machos e fêmeas; número total de animais: 142 ratos.

Atenciosamente,

Profª Tania Rieger
Presidente do CEUA/CCB-UFPE
SIAPE 2306924