UNIVERSIDADE FEDERAL DE PERNAMBUCO – UFPE

DAVID FILIPE DE SANTANA

Repercussões morfofuncionais de uma restrição dietética em ácidos graxos essenciais sobre neurônios GABAérgicos e astrócitos no sistema nigroestriatal de ratos.

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Co-Orientador: Prof. Dr. Marcelo Cairrão Araújo Rodrigues

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RECIFE

2013

A vida, minha orientadora, meus familiares, minha namorada, aos meus amigos irmãos...
Companheiros de todos os momentos e circunstâncias ...

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"Como muitas pessoas, tive a opção de fazer escolhas diversas. Contudo, escolhi seguir os caminhos científicos, tentando assim decifrar alguns enigmas que perpassam o senso comum humano . Junto com outras pessoas obtemos alguns resultados, que é desfrutar de um poder inigualável que é possuir um pouco desse vasto conhecimento que a natureza tem para nos oferecer, tentando a cada dia compreender fenômenos que embelezam e intrigam as nossas vidas. Entramos em contato com mentes sublimes e audaciosas e aprendemos que no meio cientifico, nunca chegaremos ao final e sim ao novo começo, pois como pensava o grande filósofo Nietzsche , não existem fatos eternos assim como não há verdades absolutas ..."

(SANTANA, *D.F*, 2013)

"...Aprendi que todo o mundo quer viver em cima da montanha, sem saber que a verdadeira felicidade está na forma de subir a encosta. (...) Aprendi que um homem só tem direito a olhar outro de cima para baixo quando vai ajudá-lo a levantar-se."

(GABRIEL GARCIA MARQUES)

RESUMO

Os ácidos graxos essenciais exercem um papel crucial para o desenvolvimento e manutenção do sistema nervoso. Estudos recentes do nosso laboratório têm demonstrado que a restrição dietética crônica destes macronutrientes é capaz de induzir sinais de degeneração e perda de neurônios dopaminérgicos na substantia nigra (SN) de ratos. O presente estudo visa testar a hipótese de que esta restrição dietética é capaz de alterar a distribuição de neurônios GABAérgicos nigrais bem como aumentar a funcionalidade e reatividade de astrócitos na SN e corpo estriado (CE). Ratos Wistar foram divididos em dois grupos de acordo com a dieta materna, a qual foi fornecida a partir do acasalamento e mantida por duas gerações. O grupo controle recebeu dieta balanceada contendo óleo de soja como fonte lipídica; grupo deficiente em ácidos graxos essenciais (DAGEs) recebeu dieta semelhante, mas tendo o óleo de coco como fonte lipídica. A capacidade funcional da glia foi analisada em homogenados do CE e SN a partir da atividade da enzima Glutamina Sintetase (GS). A distribuição dos astrócitos e neurônios GABAérgicos foi analisada a partir de imunohistoquímica utilizando-se anticorpos monoclonais para a Proteína Ácida Fibrilar Glial (GFAP) e parvalbumina, respectivamente. Os níveis protéicos de GFAP foram analisados por *Western blot*. O perfil de ácidos graxos essenciais em fosfolipídios da SN ou CE foi analisado por cromatografia gasosa. Análise do padrão de complexidade morfológica dos astrócitos foi realizada avaliando-se índices como dimensão fractal, área de arborização e lacunaridade. Os resultados obtidos evidenciaram um aumento significativo ($p < 0.01$) na atividade da GS no CE de animais jovens (30-40 dias) e adultos (90-110 dias) do grupo DAGES, comparado ao controle. Na SN, um aumento na atividade da GS do grupo DAGES ($p<0.001$) só foi observado aos 90 dias. Um aumento na dimensão fractal dos astrócitos ($p=0.009$) foi observado apenas nos animais jovens do grupo DAGES, comparado ao controle. No entanto um aumento da área de arborização dos astrócitos foi detectado no grupo DAGES tanto em animais jovens ($p=0.004$) como nos adultos ($p=0.005$). Não houve diferença na lacunaridade tanto nos animais jovens quanto nos animais adultos. Modificações na expressão das isoformas da proteína GFAP foram também induzidas pela dieta experimental nos animais jovens, observando-se um aumento na isoforma de 42 KDa no CE, SN assim como no córtex cerebral. Análise dos astrócitos marcados para GFAP sugere ausência de astrogliose na SN do grupo DAGEs tanto em animais jovens como adultos. Quantificação preliminar realizada em 4 animais adultos do grupo DAGES e 3 animais do grupo controle sugere que o número de neurônios imunorreativos a parvalbumina da SN não difere entre os grupos. Os resultados obtidos sugerem que a restrição dietética em ácidos graxos essenciais por duas gerações induz efeitos adversos sobre a diferenciação e atividade funcional dos astrócitos, evidenciando também uma reatividade diferenciada entre as células da glia da SN e CE. Contudo, a restrição dietética por duas gerações não é capaz de causar astrogliose na SN dos animais jovens e adultos.

Palavras-chaves: Reatividade glia, ácido docosahexaenóico, ácido araquidônico, parvalbumina, substantia nigra, corpo estriado

ABSTRACT

Essential fatty acids (EFA) play a crucial role in the development and maintenance of the nervous system. Recent studies in our laboratory have shown that chronic dietary restriction of these macronutrients are able to induce signs of degeneration and loss of dopaminergic neurons in the substantia nigra (SN) of rats. This study aims to test the hypothesis that EFA dietary restriction can alter the distribution of nigral GABAergic neurons and increase morpho-functional astrocytic reactivity in the SN and corpus striatum (CS). Wistar rats were divided into two groups according to maternal diet, which was supplied from the mating and maintained for two generations. The control group (C) received balanced diet containing soybean oil as lipid source and essential fatty acids deficient group (EF2) received a diet containing coconut oil as lipid source. The functional capacity of glia was analyzed in homogenates of CS and SN by glutamine synthetase (GS) enzymatic activity. The distribution of GABAergic neurons and astrocytes was analyzed using an immunohistochemistry approach with monoclonal antibodies to glial fibrillary acidic protein (GFAP) and parvalbumin, respectively. GFAP protein levels were analyzed by Western blot. The profile of essential fatty acids in SN or CS phospholipids was analyzed by gas chromatography. Analysis of astrocyte morphological complexity was carried out assessing the fractal dimension, arbor area and lacunarity. The results showed a significant ($p < 0.01$) in GS activity in the CS of young animals (30-40 days) and adult (90-110 days) EF2 groups, when compared to control. In the EF2 group SN an increase in GS activity ($p < 0.001$) was observed only at 90 days. A significant increase in the astrocyte fractal dimension was found in EF2 young animals, as compared to control. However, increased astrocyte arborization area was detected in both young and adult animals. There was no intergroup difference in the lacunarity either in young or adult animals. Changes in the expression of GFAP protein isoforms were also induced experimental diet in young animals, characterized by an increased expression in the 42 kDa isoform in both CS and SN. Analysis of the number of GFAP+ astrocytes suggests absence of astrogliosis in the SN of animals fed the experimental diet, in the two ages analyzed. Preliminary quantification carried out in 4 EF2 adult animals and 3 control animals, suggests that the number of GABAergic neurons containing parvalbumin did not differ to that found in the control. The results suggest that EFA dietary restriction over two generations induces adverse effects on the differentiation and functional activity of astrocytes, showing also a differential reactivity between the glial cells of the SN and CS. However, this type of dietary restriction was not able to induce astrogliosis in the SN of young and adult animals.

Keywords: astrocytes; docosahexaenoic acid; arachidonic acid; substantia nigra; corpus striatum; parvalbumin

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- ✓ **6 – OHD** – 6 – Hidroxidopamina
- ✓ **AA –** Ácido Araquidônico
- ✓ **AGPI – CL –** Ácidos Graxos Poliinsaturado de Cadeia Longa
- ✓ **ATP –** Adenosina Trifosfato
- ✓ **BDNF –** Fator Neurotrófico Derivado do Cérebro
- ✓ **CE –** Corpo Estriado
- ✓ **DAGES** – Grupo Deficiente em Ácidos Graxos Essenciais
- ✓ **DHA** – Ácido Docosahexaenóico
- ✓ **DPA** - Docosapentaenóico
- ✓ **EPA -** Ácido Eicosapentanóico
- ✓ **GABA** – Ácido γ – aminobutírico
- ✓ **GAD** –Glutamato Descarboxilase
- ✓ **GDNF –** Fator neurotrófico derivado da glia
- ✓ **GFAP** - Proteína Glial Fibrilar Ácida (GFAP)
- ✓ **GPx –** Glutationa Peroxidase
- ✓ **GR –** Glutationa Redutase
- ✓ **GS –** Glutamina Sintetase
- ✓ **GSH –** Glutationa Reduzida
- ✓ **GSSG –** Glutationa Oxidada
- ✓ **MPTP –** 1 – metil – 4 – fenil – 1,2,3,6 - tetrahidroxipiridina
- ✓ **SN –** Substância Negra

SUMÁRIO

1. INTRODUÇÃO

1.1 A importância nutricional dos lipídios durante o desenvolvimento do Sistema Nervoso Central (SNC)

Os lipídeos estão entre os principais macronutrientes envolvidos no desenvolvimento do organismo, tendo um papel muito importante no processo evolutivo de diversas espécies, inclusive a humana. Alguns estudos evidenciam que um importante marco na evolução do cérebro de humanos foi a descoberta de alimentos de alta qualidade, ricos em ácidos graxos essenciais, originados de fontes marinhas ou de água doce (CARLSON, S. J. *et al.*, 2013). A exploração destes alimentos ao longo de várias gerações coincidiu com a rápida expansão da substancia cinzenta do córtex cerebral, o que caracteriza o cérebro do homem atual (CRAWFORD *et al.*, 1999; BRADBURY, 2011). Os ácidos graxos essenciais pertencem às famílias ω-3 e ω-6, cujos precursores são o ácido α-linolênico (C18:3n-3) e o ácido linoléico (C18:2n-6) respectivamente.

O ácido α-linolênico é precursor de ácidos graxos poliinsaturados de cadeia longa (AGPI-CL) dos quais o docosahexaenóico (DHA) e o ácido eicosapentanóico (EPA) são os que apresentam maior atividade biológica (CARLSON, S. E., 2001). O DHA é o mais abundante AGPI-CL no cérebro e é utilizado pelas células neurais tanto para o desenvolvimento quanto para as suas atividades funcionais (CALDERON e KIM, 2004; MCNAMARA e CARLSON, 2006).

Já a família ômega – 6 derivada do ácido linoléico dá origem ao ácido araquidônico (AA) que é um importante componente das membranas celulares de todas as células do organismo (CARLSON, 2002). A síntese destes AGPI-CL depende da atividade de enzimas dessaturases e alongases. Em mamíferos, estas enzimas são encontradas principalmente no fígado, placenta e no sistema nervoso, são restritas aos astrócitos e endotélio vascular. Nestes últimos, a capacidade de sintetizar o DHA é essencial para o funcionamento adequado dos neurônios (DUTTA-ROY, 2000; WILLIARD *et al.*, 2001). A **figura 1** mostra o metabolismo desses ácidos graxos essencias.

Uma nutrição adequada deve fornecer níveis balanceados destes ácidos graxos essenciais, considerando que os mesmos podem exercer ações opostas no organismo devido

aos seus metabólitos, como: prostaglandinas, leucotrienos e tromboxanos, denominados de eicosanóides, alguns dos quais sendo derivados da série ômega-3 podem ter características anti inflamatórias, neuroprotetoras e sinaptogênicas (neuroprotectinas, resolvinas e sinapsinas). Parte daqueles que são decorrentes do metabolismo da séria ômega-6 podem atuar como agentes pró inflamatórios como mostrado na **figura 1** abaixo (CHIN *et al.*, 1995; KIM, 2007; SCHMITZ e ECKER, 2008; SERHAN *et al.*, 2008; BOUSQUET *et al.*, 2011).

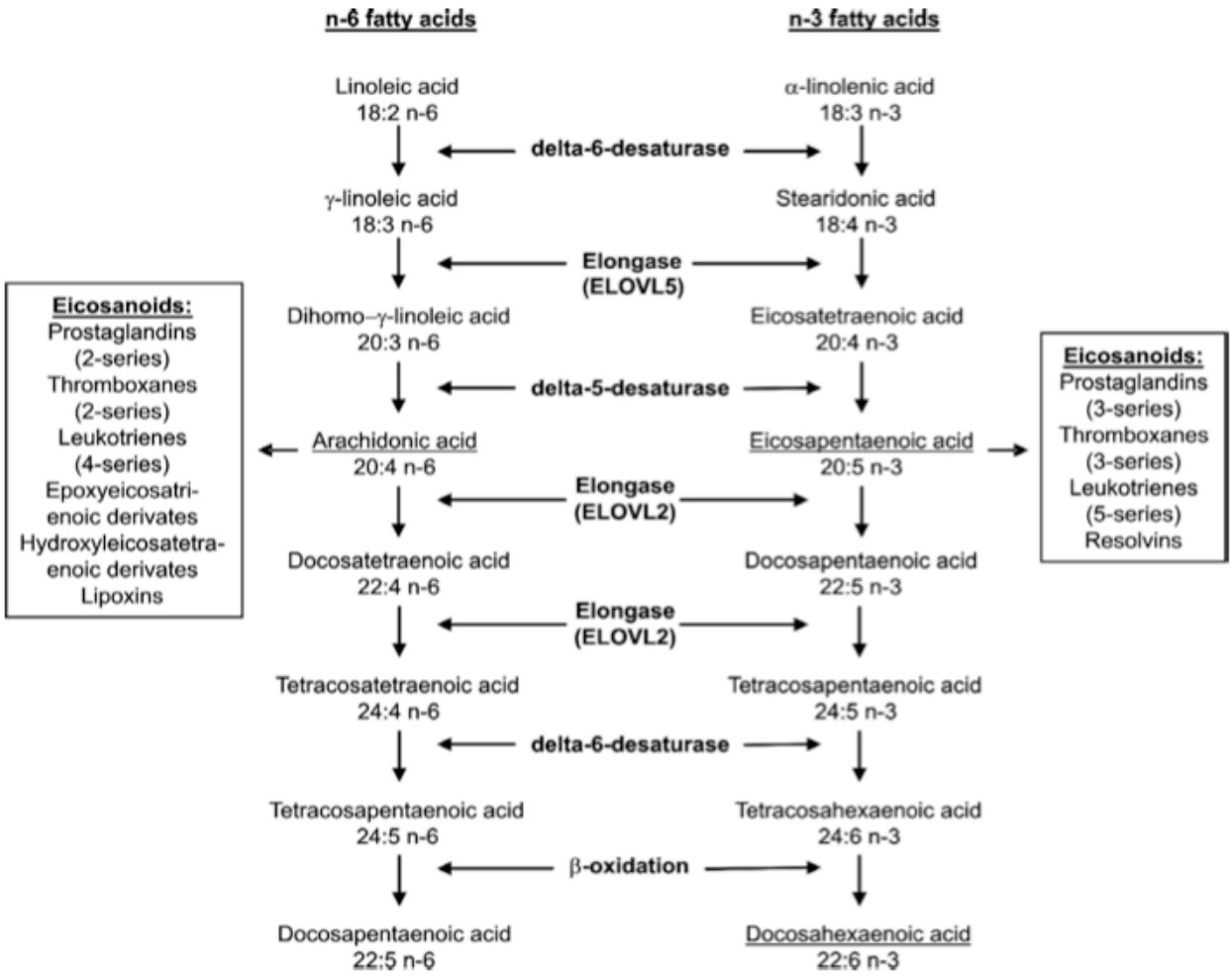

Figura 1. Metabolismo dos ácidos graxos das famílias ômega 3 e ômega 6. (SCHMITZ e ECKER, 2008)

Logo, um desequilíbrio nos níveis desses ácidos particularmente do ômega-3 pode levar a mudança no metabolismo cerebral com diminuição das respostas anti-inflamatórias e antioxidantes induzindo sofrimento neuronal (OZSOY, SEVAL-CELIK*, et al.*, 2011).

Em tempos mais remotos, como no período paleolítico, nossa alimentação caracterizava-se pelo alto consumo de alimentos de origem marinha rico em ômega-3 o que proporcionavam uma razão equilibrada de 2:1 a 1:1 (ômega-6/ômega-3) e o menor risco para algumas doenças neurodegenerativas (EATON *et al.*, 1998; SIMOPOULOS, 2011). Contudo,

atualmente estudos vêm mostrando um desequilíbrio nessa relação principalmente nos países ocidentais, caracterizando-se pelo consumo de alimentos ricos em ômega-6, e pobres em ômega-3 o que pode está associado ao surgimento de algumas patologias em vários sistemas orgânicos (AILHAUD *et al.*, 2006; BRADBURY, 2011; SIMOPOULOS, 2011).

Os dois períodos mais críticos para acumulação dos ácidos graxos essenciais (AGEs) no sistema nervoso são durante o desenvolvimento fetal e após o nascimento, durante o período de aleitamento. Durante o período gestacional o sistema enzimático fetal ainda é muito incipiente e possui baixa atividade. As enzimas alongases e dessaturases são encontradas na placenta e por isso a mesma tem um papel essencial na acumulação e transferência dos AGPI-CL para o feto (INNIS, 2005; GIL-SANCHEZ *et al.*, 2011).

Logo após a síntese ou captação pelo cérebro, a distribuição e o acréscimo de DHA ocorre em taxas variáveis e em diferentes etapas do desenvolvimento (FAVRELIERE *et al.*, 1998; CARRIE *et al.*, 2000; DIAU *et al.*, 2005). Algumas regiões têm uma maior concentração de DHA como o córtex, cerebelo e estriado e outras uma menor como o hipotálamo, mesencéfalo e a medula oblonga (XIAO *et al.*, 2005). Por isto, mudanças na composição da dieta afetam diferentemente as áreas cerebrais bem como comprometem o envelhecimento natural do cérebro (MARTEINSDOTTIR *et al.*, 1998; XIAO, HUANG *et al.*, 2005). Por esses motivos, nos últimos anos muitos estudos realizados tanto em humanos quanto em animais experimentais têm associado níveis inadequados de DHA a doenças neurodegenerativas e psiquiátricas (SUMIYOSHI *et al.*, 2008; PALSDOTTIR *et al.*, 2012).

1.2 **Efeitos dos derivados do DHA no sistema nervoso central**

Durante o desenvolvimento do sistema nervoso, os AGPI-CL exercem importantes funções em diversos processos, tais como: sinalização intracelular (DE WILDE *et al.*, 2002; GUPTA *et al.*, 2012), neurotransmissão (DELION, D. S. *et al.*, 1994; ZIMMER *et al.*, 2000; CHALON, 2006), mecanismos anti apoptóticos (EADY *et al.*, 2012) e neuroproteção (BELAYEV *et al.*, 2009; ZHANG, C. e BAZAN, 2010), mielinização (NAVE, 2010), funções cognitivas e nível de atenção (JIANG *et al.*, 2009; CHIU *et al.*, 2012; YEHUDA, 2012), comportamento motor e emocional (FEDOROVA e SALEM, 2006; SHARMA *et al.*, 2012) plasticidade sináptica (DE VELASCO *et al.*, 2012), sinaptogênese (KIM, SPECTOR, *et al.*, 2011), diferenciação de neurônios (CALDERON e KIM, 2004) e de astrócitos (JOARDAR *et al.*, 2006).

Grande parte destas ações são devidas à participação direta do DHA ou de derivados deste ácido graxo. Na retina, por exemplo, os fotorreceptores utilizam o DHA como precursor de

neuroprotectina D1 (NPD1), que é obtida pela oxigenação seletiva do DHA pela 15 – Lipoxigenase (15-LOX-1). A síntese de NPD1 é induzida por injúria, estresse oxidativo, neurotrofinas, isquemia e reperfusão e pela proteína precursora da beta amilóide (CALANDRIA *et al.*, 2009). Logo, em resposta aos insultos, ativação de NPD1 desencadeia respostas sinalizadoras de proteção via diversos mecanismos, como por exemplo o aumento das proteínas anti-apoptóticas (Bcl-2 e Bcl-xL) e a diminuição de proteínas pró- apoptóticas (Bax e Bad) (MARCHESELLI *et al.*, 2003; MUKHERJEE *et al.*, 2004; LUKIW *et al.*, 2005).

Outro derivado do DHA é o N-docosahexaenoil Etanolamida (DEA) que é uma forma amida do docosahexaenóico que atua em diversas funções importantes no cérebro (KIM, SPECTOR *et al.*, 2011). Alguns estudos mostram que o DEA aumenta o crescimento de neuritos e induz sinaptogênese no hipocampo (KIM, MOON*, et al.*, 2011). Esse derivado do DHA também atua em uma família de fosfoproteínas especificas de neurônios que são as sinapsinas, as quais estão associadas às vesículas sinápticas e atuam na sinaptogênese, maturação e função sináptica assim como na liberação de neurotransmissores (LU *et al.*, 1992; CHIN, LI *et al.*, 1995; BALDELLI *et al.*, 2007).

1.3 **Efeitos da Restrição e Suplementação com AGEs sobre o SNC**

Vários estudos têm demonstrado que reduções na concentração de ácido α – linolênico na dieta, durante o período perinatal, estão associadas a alterações na neurotransmissão GABAérgica (HAMANO *et al.*, 1996), glutamatérgica (MOREIRA *et al.*, 2010), colinérgica (AID *et al.*, 2003) e monoaminérgica (DELION, INSOLIA *et al.*, 1994; CHALON, 2006). O sistema dopaminérgico é extremamente vulnerável a este tipo de deficiência nutricional, tendo sido relatado que o mesmo reduz níveis de dopamina no núcleo *accumbens*, córtex frontal (ZIMMER *et al.*, 2000; (ZIMMER, DELION-VANCASSEL *et al.*, 2000; ZIMMER *et al.*, 2002) e corpo estriado (CE) (BOUSQUET *et al.*, 2008) de ratos, o que provoca alterações comportamentais indicativas de comprometimentos nestes sistemas monoaminérgicos (LEVANT *et al.*, 2004) bem como diminui o número de neurônios dopaminérgicos na porção compacta da substância negra (SN) (AHMAD *et al.*, 2008; PASSOS *et al.*, 2012).

Por outro lado, a suplementação dietética com óleo de peixe rico em EPA e DHA é capaz de reduzir vários efeitos deletérios induzidos por insultos oxidativos ou inflamatórios em regiões envolvidas com o sistema dopaminérgico, como os núcleos da base. A **quadro 1** resume dados de vários artigos que avaliaram os efeitos da restrição ou suplementação de AGES sobre aspectos relativos ao sistema dopaminérgico e núcleos da base.

Tabela. 1 Efeitos da restrição e suplementação dos AGEs sobre o sistema nervoso central

Restrição	Efeitos	Referências
Ácido α – linolênico	↓ a densidade do receptores D2 e concentração de dopamina	(DELION, S. *et al.*, 1994)
Ácido α – linolênico	↓ Atividade dopaminérgico mesolímbica	(ZIMMER, DELION-VANCASSEL *et al.*, 2000)
Ácido α – linolênico	↓ vesículas dopaminérgicas	(ZIMMER, VANCASSEL *et al.*, 2002)
Ácido α – linolênico	Desregulação dopaminérgica e alterações comportamentais	(LEVANT, RADEL *et al.*, 2004)
Ácido α – linolênico	↓ dos níveis transportadores de serotonina no núcleos accumbens e no córtex frontal	(CHALON, 2006)
Ácido α – linolênico	↓ expressão de enzimas de sintese de AA e DHA no cortex	(RAO *et al.*, 2007)
Ácido α – linolênico	↓ número de neurônios dopaminérgicos na substância negra compacta	(AHMAD, PARK *et al.*, 2008)
DHA	↓ número de neurônios dopaminérgicos na substância negra compacta	(PASSOS, BORBA *et al.*, 2012)
Suplementação	**Efeitos**	**Referências**
DHA e EPA	↓ Peroxidação lipídica e da enzima superóxido dismutase (SOD) no estriado	(SARSILMAZ *et al.*, 2003)
DHA	↑ diferenciação de astrócitos corticais	(JOARDAR, SEN *et al.*, 2006)
DHA e EPA	↓ Morte de neurônios dopaminérgicos na substância negra compacta em modelo de 6 – OHD	(CANSEV *et al.*, 2008)
DHA e EPA	↓ Morte de neurônios dopaminérgicos na substância negra compacta em modelo de MPTP	(BOUSQUET, SAINT-PIERRE *et al.*, 2008)
Ácido α – linolênico	↑ Síntese de BDNF no estriado	(BOUSQUET *et al.*, 2009)
DHA e EPA	↓ Estresse oxidativo em modelo de 6 – OHD	(DELATTRE *et al.*, 2010)
DHA	↓ dos níveis e atividade da PGE – 2 e ↓ da morte dos neurônios dopaminérgicos em modelo de 6 – OHD	(OZSOY, TANRIOVER, *et al.*, 2011)

1.4 Importância das interações entre neurônios e células da glia e participação do DHA.

Já é bem evidenciado que os astrócitos estão entre as células gliais mais abundantes do sistema nervoso e muito embora o seu papel no sistema nervoso por muito anos tenha sido considerado passivo e pouco relevante para manutenção das funções neuronais, sabe-se atualmente que neurônios e células glias exercem um papel crucial para o funcionamento neuronal interagindo através de diversos mecanismos como: o metabolismo do glutamato e GABA (BAK *et al.*, 2006), tamponamento dos níveis de K^+ do meio extracelular (PARPURA *et al.*, 2012), secreção de citocinas (WIESE *et al.*, 2012), síntese de moléculas que compõe a matriz extracelular em torno das células neuronais (FAISSNER *et al.*, 2010), metabolismo de glicogênio (BROWN e RANSOM, 2007), formação da barreira hematoencefálica, captação de neurotransmissores, regulação iônica assim como estabelecendo ações que podem ocorrer de forma unilateral como por exemplo a sinaptogênese e mielinização ou de forma bilateral como a diferenciação e liberação de fatores tróficos (HAYDON, 2000; PAIXAO e KLEIN, 2010).

Hoje se sabe que existe uma relação imprescindível entre o astrócito e o neurônio pré e pós sináptico formando a sinapse tripartide (ARAQUE *et al.*, 1998; WANG e BORDEY, 2008).

Além disto, as junções comunicantes entre os astrócitos e entre alguns tipos de neurônios podem modular a transmissão sináptica (PEREA e ARAQUE, 2010). Os astrócitos liberam também uma grande variedade de gliotransmissores como o glutamato, ATP e fatores neurotróficos. Em relação aos núcleos da base, os astrócitos estão relacionados com a liberação do fator neurotrófico derivado da glia (GDNF) (LIN *et al.*, 1993; KRIEGER *et al.*, 1995; CHEN, P. S. *et al.*, 2006) fator neurotrófico derivado do cérebro (BDNF) (KNOTT *et al.*, 2002; CHEN, P. S., PENG *et al.*, 2006) e fator neurotrófico derivado de astrócito mesencefálico (MANF) (PETROVA *et al.*, 2003). Estudos já mostraram que esses fatores neurotróficos, dentre outros, são importantes para a gênese, diferenciação e proteção de neurônios dopaminérgicos (ZHANG, W. *et al.*, 2005; CHEN, P. S., PENG *et al.*, 2006). Logo, a interação bidirecional entre neurônio e glia é crucial para regulação funcional de vários aspectos envolvidos com a estrutura, função e metabolismo do sistema nervoso. A **figura** 2 ilustra algumas das funções dos astrócitos, no sistema nervoso.

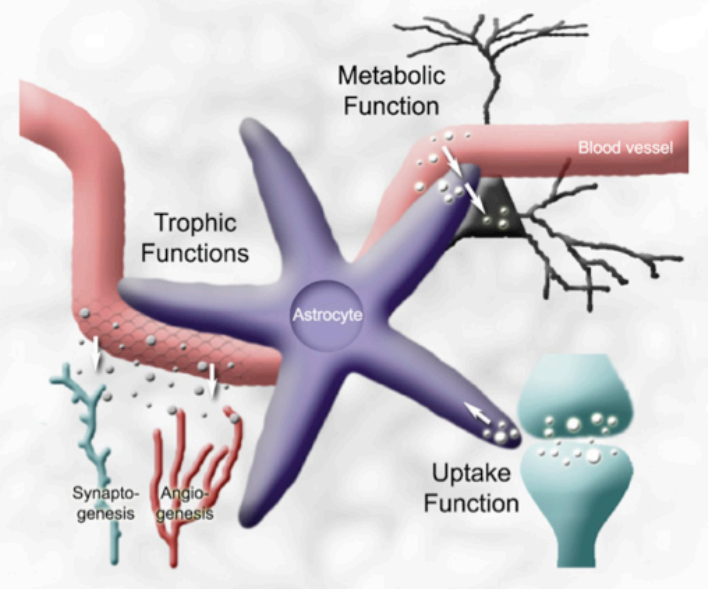

***Figura 2**. Interação Neurônio – Astrócitos na sinapse tripartide. (WANG e BORDEY, 2008)*

Dentre as funções astrocitárias, a metabolização do glutamato é vital para o funcionamento adequado dos neurônios, já que esse é o neurotransmissor excitatório mais abundante do sistema nervoso central dos vertebrados (NEDERGAARD *et al.*, 2002). Aferências glutamatérgicas são intensas na região dos núcleos da base principalmente para o CE que as recebe de todo o córtex cerebral (PARENT e HAZRATI, 1995).

Após ser liberado na fenda sináptica o glutamato atua nos seus receptores pós-sinápticos, mas também é rapidamente recaptado pelos astrócitos através de transportadores astrogliais específicos dependentes de sódio como o transportador de glutamato aspartato (GLAST), transportador de glutamato (GLT-1) e transportador de aminoácidos excitatórios (EAAC1) (SHELDON e ROBINSON, 2007).

É de grande importância que os níveis de glutamato na fenda permaneçam baixos, pois em excesso, a ação glutamatérgica pode causar excitotoxicidade aos neurônios e até aos astrócitos, a qual pode está relacionada com a etiologia de algumas neuropatologias (CHEN, C. J. *et al.*, 2000; LEE, J. *et al.*, 2011).

Após captação pelos astrócitos, o glutamato, tem destinos diversos podendo ser utilizado como substrato no ciclo de Krebs, na síntese de glutationa que é a molécula antioxidante mais abundante nos astrócitos e também é utilizado para síntese de glutamina através da enzima glutamina sintetase (GS), uma enzima glial (NEDERGAARD, TAKANO *et al.*, 2002). Embora neurônios também sejam capazes de sintetizar glutationa, os astrócitos desempenham um papel muito importante em suprir os substratos necessários à síntese da glutationa pelos neurônios (MARTIN e TEISMANN, 2009). Em condições homeostáticas a GS só é expressa nos astrócitos, sendo sua atividade um importante indicador da atividade astrocitária diante de várias condições fisiológicas e patológicas e é responsável por manter em condições homeostáticas os níveis de glutamato (NEDERGAARD, TAKANO *et al.*, 2002).

Durante a síntese da glutamina, a GS utiliza o glutamato e a amônia juntamente com o ATP, sendo o Mg^{2+} o cofator da reação como esquematizado na **figura 3**.

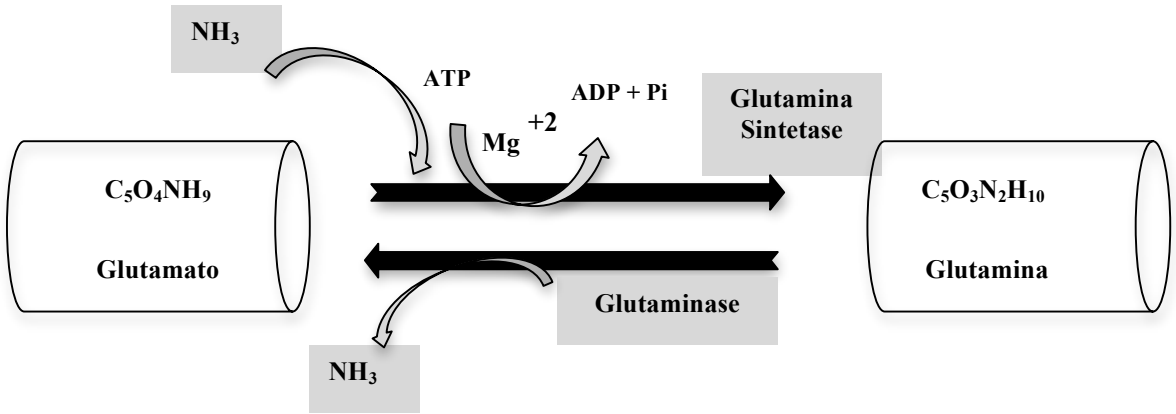

Figura 3. Equação envolvida na metabolização do glutamato e amônia para síntese da glutamina. Mg^{+2} (Magnésio). NH_3 (Amônia) ATP (Adenosina Trifosfato), ADP (Adenosina Difosfato), Pi (Fosfato)

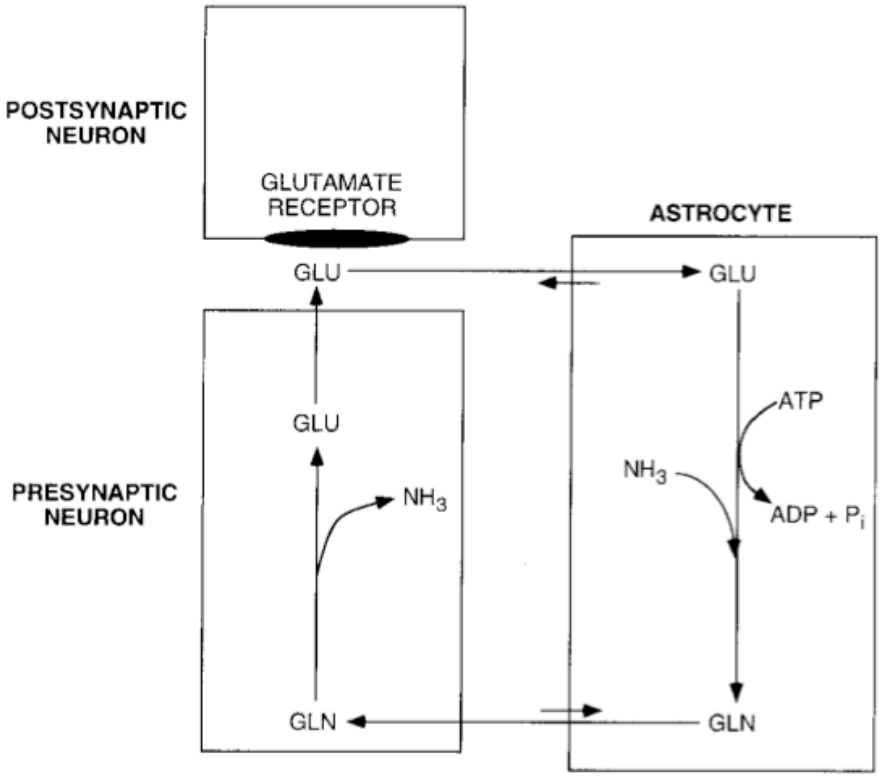

Figura 4. O ciclo da glutamina no cérebro. GLU (Glutamato). GLN (Glutamina) (COOPER, 2001)

Assim a atividade da GS tem um papel de desintoxicação do sistema nervoso, pois em excesso, tanto o glutamato quanto a amônia são maléficos para o tecido neuronal (KOSENKO *et al.*, 2003; LLANSOLA *et al.*, 2007; LEE, J., YIM *et al.*, 2011). Um aumento na eficácia da GS está relacionado com a neuroproteção (ZOU *et al.*, 2011). Alguns fatores são capazes de reduzir a atividade da GS, como por exemplo, níveis elevados de óxido nítrico e a ativação do receptor glutamatérgico do tipo NMDA (KOSENKO, LLANSOLA *et al.*, 2003; RODRIGO e FELIPO, 2007).

Logo, os níveis e a atividade da GS são considerados bons indicadores para avaliar a atividade de astrócitos como uma resposta a lesão neuronal, podendo estar relacionados a situações patológicas e de reatividade glial (PETITO *et al.*, 1992; HABERLE *et al.*, 2011). A **figura 4** ilustra a interação neurônio-glia envolvida na metabolização do glutamato e amônia e síntese da glutamina.

1.5 Reatividade astrocitária em condições neurodegenerativas que envolvem os núcleos da base.

Já é bem estabelecido que após insultos ou lesão no sistema nervoso os astrócitos tornam-se reativos e respondem peculiarmente de várias formas, sendo esta resposta conhecida como gliose reativa ou astrogliose (MCGEER e MCGEER, 2008). Tal resposta pode estar associada à proliferação e/ ou hipertrofia glial podendo ocorrer durante o curso neurodegerativo como mostrado em modelo de doença de parkinson (DERVAN *et al.*, 2004; BARRES, 2008). No entanto, se a proliferação astrocitária protege ou exacerba a perda de neurônios na substancia negra, é ainda muito debatido (MYTHRI *et al.*, 2011).

Durante o processo de proliferação e hipertrofia, os astrócitos reativos aumentam a expressão da proteína ácida fibrilar glial (GFAP) que é um filamento intermediário tipo III (ENG *et al.*, 2000). Esta proteína pode apresentar quatro isoformas, sendo a isoforma fosforilada principal caracterizada pelo peso molecular de 50 kD. Modificações na expressão das diversas isoformas de GFAP podem ocorrer através de oxidação, acetililação sulfonação e fosforilação (NOETZEL, 1990). A expressão de isoformas de menores pesos moleculares foram observadas em tumores gliais (LUIDER *et al.*, 1999). Já foi demostrado que em condições de reatividade glial há um aumento na expressão da banda fosforilada (50 KDa) diante de insulto hipóxico-isquêmico (SULLIVAN *et al.*, 2012). A maturidade astrocitária também envolve aumento na expressão da isoforma fosforilada de GFAP (JOARDAR e DAS, 2007). Alguns estudos in vitro relatam que a fosforilação da proteína GFAP promove desagregação de filamentos intermediários enquanto que a sua desfosforilação por fosfatases recupera a sua habilidade para polimerizar (INAGAKI *et al.*, 1990). Estudos *in vitro* utilizando astrócitos do córtex cerebral mostraram que quando os mesmos são colocados em um meio rico em fatores tróficos e hormônios, adquirem um fenótipo reativo e a expressão da banda não-fosforilada também aumenta (GRINTAL *et al.*, 2009).

Experimentos em ratos mostraram que após lesão de neurônios dopaminérgicos da substância negra pars compacta com 6-OHDA, há um aumento nas células GFAP positivas e um efeito neuroprotetor do sistema nigroestriatal quando os animais são colocados em um ambiente enriquecido com alguns objetos, tais como cones, bolas, cubos, rampas, prateleiras e escadas (ANASTASIA *et al.*, 2009). Em modelos experimentais de doença de Parkinson, a ativação astrocitária continua mesmo após morte dos neurônios dopaminérgicos da porção compacta da substância negra (KOHUTNICKA *et al.*, 1998) (PRZEDBORSKI *et al.*, 2000). Em algumas condições, a resposta reativa exacerbada dos astrócitos pode aumentar a liberação de citocinas pró-inflamatórias como a interleucina β e fator de necrose tumoral α (GIULIAN *et al.*, 1988).

A importância do DHA sobre estrutura e função de astrócitos vem sendo demonstrada em alguns trabalhos *in vitro*. Joardar *et al.*,(2006), por exemplo, demonstraram que o DHA está envolvido na diferenciação ´morfológica dos astrócitos favorecendo a expansão dos seus prolongamentos. Em tal ação o DHA atua favorecendo a atividade de receptores beta-adrenérgicos. Em culturas primárias de astrócitos corticais foi também demonstrado que o tratamento dos mesmos com ácidos graxos advindos do óleo de coco, que contém baixos níveis de DHA, comprometem a diferenciação morfológica dos mesmos, mantendo-os com poucos prolongamentos e um fenótipo similar ao de glia radial (JOARDAR e DAS, 2007). Neste mesmo trabalho também foi observada a expressão de várias isoformas de GFAP com pesos moleculares menores do que 50 KDa (JOARDAR e DAS, 2007). Tem sido demonstrado que DHA exerce efeito protetor sobre a viabilidade de astrócitos corticais de hamsters mantidos em cultura (CHAMPEIL-POTOKAR *et al.*, 2004) bem como promove efeito estimulatório sobre a distribuição funcional das junções Gap presentes neste astrócitos (CHAMPEIL-POTOKAR *et al.*, 2006).

Estudos *in vitro* também demonstraram que DHA exerce uma atividade modulatória sobre a captação de glutamato pelos astrócitos. Enquanto é capaz de estimular a atividade dos transportadores GLT1 e EAAC1, em condições fisiológicas o DHA inibe a atividade do GLAST em células embrionárias renais (BERRY *et al.*, 2005) e astrócitos corticais (GRINTAL, CHAMPEIL-POTOKAR *et al.*, 2009). No entanto sob condições de reatividade astrocitária, o DHA não inibe a captação do glutamato (GRINTAL, CHAMPEIL-POTOKAR *et al.*, 2009).

Embora todos estes estudos tenham sido realizados *in vitro*, os efeitos relatados contribuem para modificar as interações neurônio-glia, uma vez que o acoplamento e a diferenciação de astrócitos são importantes para neuroregulação e neuroproteção. Em um trabalho recente realizado in vivo, foi demostrado que em animais adultos submetidos a uma restrição dietética em alfa-linolênico desde o período gestacional, não há sinais de astrogliose no hipocampo (LATOUR *et al.*, 2013). No entanto, no hipocampo, a deficiência dietética em ômega-3 por uma ou duas gerações não induz redução no número de neurônios (AHMAD, PARK *et al.*, 2008).

Evidências recentes obtidas no laboratório de Neurofisiologia da UFPE mostraram que animais jovens submetidos a uma restrição dietética em AGES por duas gerações, apresentam uma perda de 20% no número de neurônios dopaminérgicos da substância negra rostro-dorso-medial (PASSOS, BORBA *et al.*, 2012). Um dos potenciais mecanismos relacionados a esta perda é o estresse oxidativo observado na substancia negra destes animais (CARDOSO *et al.*, 2012). Contudo, ainda não se conhece de que forma os astrócitos do sistema nigro-

estriatal estão reagindo diante da neurodegeneração dopaminérgica induzida por este modelo experimental.

1.6 Importância dos Neurônios gabaérgicos parvalbumina positivos no sistema nigroestriatal.

A maior parte das sinapses inibitórias do encéfalo maduro usam como neurotransmissor o ácido ϒ – aminobutírico (GABA) envolvido em diversas atividades biológicas como atividade locomotora, aprendizagem e memória, dentre outras funções (SOGHOMONIAN e MARTIN, 1998).

Logo, é muito importante a neurotransmissão GABAérgica assim como a dopaminérgica e glutamatérgica principalmente nos núcleos da base. Por isso, a morte dos neurônios dopaminérgicos da substância negra pode levar à inibição e hipoatividade do tálamo como a dificuldade no início dos movimentos (acinesia) e redução na extensão e velocidade dos movimentos (bradicinesia) sinais hipocinéticos encontrados na doença de Parkinson. Por outro lado, a morte dos neurônios GABAérgicos do estriado junto aos neurônios da substância negra pode causar uma hiperativação do tálamo, e ambas as situações levam a disfunções da alça sensoriomotor (SCHINTU *et al.*, 2009).

Na substância negra a maior parte dos neurônios são GABAérgicos ou dopaminérgicos, embora populações menores de neurônios nitrérgicos e colinérgicos foram também descritas (GONZALEZ-HERNANDEZ *et al.*, 1997). As células dopaminérgicas são distribuídas normalmente em duas regiões: a rostro-dorso- medial da substância negra pars compacta (SNrm) e caudo-ventro-lateral (SNcv) incluindo a substância negra pars reticula e a região ventrolateral da pars compacta (BAYER *et al.*, 1995; GONZALEZ-HERNANDEZ, BARROSO-CHINEA *et al.*, 2001).

Por outro lado os neurônios gabaérgicos estão localizados predominantemente na SN pars reticulada e lateral e em menor grau na SNrm. Estes neurônios compõem uma das vias de saída mais importantes dos núcleos da base (APPELL e BEHAN, 1990; REDGRAVE *et al.*, 1992) além de exercerem atividade modulatória sobre os neurônios dopaminérgicos locais. Há também relatos de que algumas células dopaminérgicas nigrais da região reticulada coexpressam a enzima glutamato descarboxilase (GAD), que é a enzima de síntese do GABA, (CAMPBELL *et al.*, 1991; RODRIGUEZ e GONZALEZ-HERNANDEZ, 1999; GONZALEZ-HERNANDEZ, BARROSO-CHINEA *et al.*, 2001).

As células GABAérgicas expressam duas isoformas da GAD, com peso molecular de 65 ou 67 KDa as quais apresentam uma distribuição e atividade funcional distinta em várias regiões do cérebro (ERLANDER e TOBIN, 1991; FUKUDA *et al.*, 1997; SOGHOMONIAN e MARTIN, 1998). Na substância negra a GAD_{67} está localizada principalmente na pars reticulata, um pouco na região lateral e na pars compacta. Por outro lado a GAD_{65} é muito expressa na região rostro-dorso-medial (GONZALEZ-HERNANDEZ, BARROSO-CHINEA *et al.*, 2001).

De acordo com Gonzalez- Hernández (2001) existem projeções que saem da Substancia negra pars reticulada expressando GAD_{67}, com o objetivo de manter os níveis de GABA para as atividades neurais duradouras, diferentemente da projeção que sai da SN compacta e expressa a GAD_{65} que sintetiza o GABA diante de demandas locais. A **figura 5** ilustra dois tipos de aferências GABAérgicas nigroestriatais, de acordo com Gonzalez-Hernández (2001).

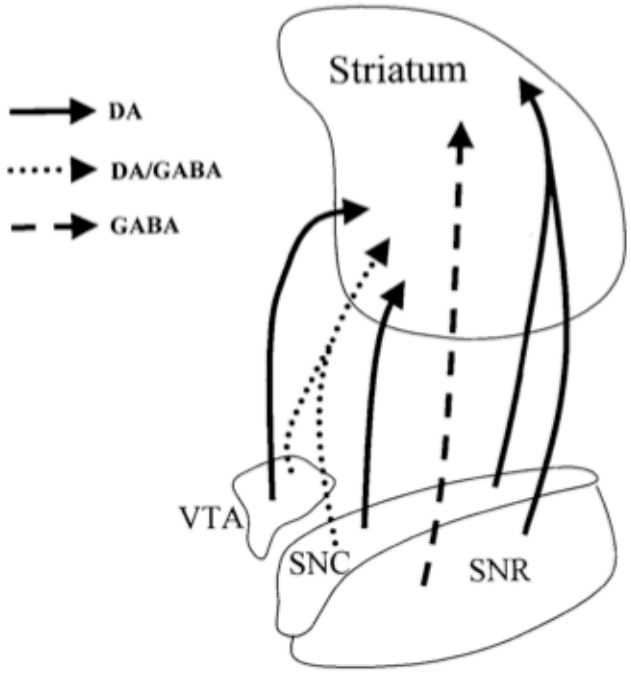

Figura 5. (Três vias mesoestriatais: A via dopaminérgica que surge de núcleos mesencefálicos, a via GABAérgica que surge na SNR e expressam GAD_{67} e a via dopaminérgica/GABAérgica (DA/GABA) que surge a partir de neurônios dopaminérgicos na região medial da SNC e Área Tegmentar Ventral (VTA) que expressam a GAD_{65}) (GONZALEZ-HERNANDEZ *et al.*, 2001).

Além das projeções para o estriado, há também projeções GABAérgicas que saem da SN pars reticulada que a princípio tem como alvos principais o tálamo e o colículo superior (SIDIBE *et al.*, 2002).

Estudos eletrofisiológicos mostraram que as aferências GABAérgica provinda da SNR e SNC inibem fortemente os neurônios dopaminérgicos da SNC, podendo modular também a atividade nigro-estriatal (CHEVALIER *et al.*, 1981; NG e YUNG, 2000; SAITOH *et al.*, 2004;

TEPPER e LEE, 2007). Portanto, os neurônios GABAérgicos tem um papel muito importante na regulação intrínseca e extrínseca da substância negra, pois atuam tanto na via nigroestriatal como na estriatonigral modulando a ação dopaminérgica nestas duas regiões (REID *et al.*, 1990; TEPPER e LEE, 2007).

Uma parte dos interneurônios GABAérgicos da SN e CE expressam a proteína parvalbumina (PV) que assim como a calbindina e calretinina são proteínas moduladoras de cálcio (TEPPER e BOLAM, 2004). Calretinina (CR) e calbindina (CB) estão presentes em populações de células dopaminérgicas nigrais, enquanto a parvalbumina está presente em células GABAérgicas. Algumas células GABAérgicas localizadas na SN pars reticulata co-expressam PV e calretinina (LEE, C. R. e TEPPER, 2007). No entanto, uma análise destas células mostrou que as mesmas não diferem do ponto de vista eletrofisiológico e de projeções (LEE, C. R. e TEPPER, 2007).

Além de caracterizar populações distintas de células na SN, a presença da parvalbumina em neurônios pode conferir aos mesmos uma menor vulnerabilidade a lesão, considerando que a mesma está envolvida com o tamponamento do Ca^{+2} intracelular. Em condições homeostáticas, a PV se liga ao íon Mg^{+2}, mas sob concentração aumentada de Ca^{+2}, a PV libera o íon Mg^{+2} e se liga aos íons de Ca^{+2} evitando seus efeitos deletérios (BRAUN, 1990; PARENT *et al.*, 1996).

Este tamponamento do cálcio é muito importante para manter os níveis desse íon em condições homeostáticas pois a elevação descontrolada deste íon leva à ativação excessiva das células, injúria e consequentemente à morte celular (SHELDON e ROBINSON, 2007); (BEZPROZVANNY, 2009; CELSI *et al.*, 2009). Diminuição na atividade de tamponamento do cálcio e de neurônios contendo PV vem sendo associada a algumas doenças degenerativas como por exemplo a doença de Alzheimer (SATOH *et al.*, 1991) e a doença de Huntington (SETO-OHSHIMA *et al.*, 1988; KIYAMA *et al.*, 1990; SATOH, TABIRA *et al.*, 1991; HEIZMANN, 1993), está ultima envolvida com os núcleos da base.

2. JUSTIFICATIVA E HIPÓTESE DO PRESENTE ESTUDO

Já se sabe que o DHA é capaz de modular a atividade GABAérgica, reduzindo a atividade inibitória e potencializando a atividade excitatória através dos receptores NMDA na SN (HAMANO, NABEKURA *et al.*, 1996). Como descrito anteriormente, uma restrição dietética em AGES por uma ou duas gerações é capaz de induzir a morte de neurônios dopaminérgicos na SN (PASSOS, BORBA *et al.*, 2012) aumentando os níveis de peroxidação lipídica e reduzindo as reservas anti-oxidantes (CARDOSO, PASSOS *et al.*, 2012).

Considerando estas evidências e buscando entender potencias mecanismos envolvidos com estes efeitos deletérios sobre o sistema nigro-estriatal resolvemos investigar se tais efeitos estendem-se aos neurônios GABAérgicos e à capacidade reativa dos astrócitos, os quais normalmente atuam para minimizar o dano neuronal diante de processos degenerativos.

Assim, o presente estudo testou a hipótese de que uma restrição dietética em ácidos graxos essenciais por duas gerações é capaz de induzir reatividade morfofuncional e proliferação em astrócitos do sistema nigroestriatal e diminuir o número de neurônios GABAérgicos da substância negra de ratos jovens e adultos.

3. OBJETIVOS

3.1 Geral:

Investigar as repercussões de uma restrição dietética em AGES por duas gerações sobre a funcionalidade dos astrócitos e distribuição de neurônios GABAérgicos do sistema nigro-estriatal em ratos Wistar jovens e adultos

3.2 Específicos:

- ✓ Avaliar a atividade da enzima glutamina sintetase na substância negra e corpo estriado;
- ✓ Analisar o número de astrócitos imunorreativos para a proteína GFAP na substância negra;
- ✓ Avaliar a reatividade astrocitária através da análise da dimensão fractal, lacunaridade e área de arborização de seus prolongamentos na substância negra.
- ✓ Quantificar o número de células GABAérgicas contendo parvalbumina da substância negra;

4. MATÉRIAS E MÉTODOS EXTENDIDO

Animais utilizados

Todos os procedimentos foram aprovados pelo Comitê de Ética em Experimentação animal da Universidade Federal Pernambuco (Processo 23076.022005/2008-71). Foram utilizadas ratas Wistar pesando 200 – 250g, mantidas através da prenhez e lactação com água e dieta *ad libitum*, ciclo claro e escuro e temperatura controlada. Os grupos foram divididos em grupo controle (C) e grupo deficiente em ácidos graxos essenciais (DAGEs) os quais receberam dietas isocalóricas (400Kcal/100g), diferenciadas apenas pela fonte lipídica: o óleo de soja na dieta controle, constituído de níveis adequados dos ácidos α-linolênico e linoleico e o óleo de coco de babaçu, na dieta experimental, que possui níveis reduzidos desses ácidos graxos. A dieta de óleo de coco continha 5% de óleo de soja com quantidades adequadas de saturados, monoinsaturados, ácidos α-linolênico e linoleico. A dieta experimental continha óleo de coco 5%, com níveis reduzidos de ácido linoleico o que corresponde a 30% do requisito mínimo recomendado na dieta para roedores proposto por Bourre et al., (1990). Serão feitos experimentos com animais de 2° geração com 30 e 90 dias para todos os experimentos realizados. Essa dieta foi preparada de acordo com (SOARES *et al.*, 1995) e submetida a transesterificação de acordo com Hartman e Lago (1973) e mostrada na **tabela 1**. O perfil de ácidos graxos nas dietas foi analisado através de cromatografia gasosa e estão apresentados na **tabela 2.** A dieta foi preparada pelo menos 2 vezes por mês e estocada e armazenadas no frigorífico a 4 º C.

Dieta Utilizada

Tabela 2
Micronutrientes da composição da Dieta (g/100g de dieta) utilizada na confecção de ração para os grupos, C (óleo de soja) e DAGEs (óleo de coco)

Ingredientes	Dieta controle	Dieta Experimental
Açúcar	21,0	21,0
Caseína	20,7	20,7
Celulose	1,8	1,8
D- Metionina	0,1	0,1
Butil Hidroxitolueno	0,001	0,001
Maizena	46,8	46,8

Mix Mineral	3,7	3,7
Mix Vitamínico [x]	0,9	0,9
Óleo de coco [y]	----	5,0
Óleo de soja	5,0	----
Kcal/ 100g	399,1	400,5

[X]Mix Vitamínico (Rhoster Ind. Com LTDA, SP, Brasil) constituintes (m%): Ácido fólico (20); Niacina (300); Biotina (2) Pantotenato de cálcio (160); Piridoxina (70); Riboflavina (60); Cloreto de Tiamina (60) Vitamina B_{12} (0.25); Vitamina K_1 (7.5). Adicionar o conteúdo (UI%) Vitamina A 40.000; Vitamina D_3 10.000; Vitamina E (750).
[Y]Mix Mineral (Rhoster Ind. Com LTDA, SP, Brasil) constituintes (m%): $CaHPO_4$ (38); K_2HPO_4 (24); $CaCO_3$ (18.1); NaF (0.1); NaCl (7.0); MgO (2.0); $MgSO_4$ $7H_2O$ (9.0); $FeSO_4$ $7H_2O$ (0.7); $ZnSO_4$ H_2O (0.5) $MnSO^+$ H_2O (0.5) $CuSO_4$ 5 H_2O (0.1); $Al_2(SO_4)3K_2SO_4$ 24 H_2O(0.02); $NaSeO_3$ 5 H_2O (0.001); Kcl (0.008).

Tabela 3
Composição dos ácidos graxos na dieta (% de ácidos graxos totais)

Ácidos Graxos	Dieta controle	Dieta Experimental
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	.16
22	0.51	0.08
23	0.07	0.02
24	0.04	nd
Total de saturados	26.01	67.29
16:1	2.72	0.06
18:1 n-9	9.36	23.51
20:1	0.24	0.16
Total Monoinsaturados	12.32	23.73
18:2n-6	55.36	8.10
18:3n-3	6.04	0.49
20:2	0.04	0.06
20:5n-3	0.03	nd
22:2n-3	0.05	0.04
22:6n-3	0.13	0.06
Total de Poliinsaturados	61.65	8.75

*nd: não detectado

Perfusão de animais para obtenção de secções encefálicas e imunohistoquímica

Sob anestesia profunda do anestésico geral enflurano volátil (via respiratória), os animais foram submetidos à perfusão transcardíaca, inicialmente com NaCl a 0,9%, seguida de paraformaldeído 4% (PA) em tampão fosfato (TF) 0,1M e pH 7,4. A perfusão foi realizada com auxílio de uma bomba de infusão contínua (Harvard Apparatus). Após a perfusão os encéfalos foram pesados em balança analítica (Marte BL320H), crioprotegidos em soluções de sacarose, 10, 20 e 30%, seccionados no plano sagital (40 μm) em criostato (Leica CM 1900, Alemanha). Foram separadas seis séries de secções encefálicas as quais foram armazenadas em freezer -20°C em solução crioprotetora contendo glicerol, etileno glicol, tampão fosfato 0,2M e água destilada para posterior procedimento imunohistoquímico.

Protocolo de Imunohistoquímica para marcação da Proteína Ácida Fibrilar Glial (GFAP) e Parvalbumina (PV).

Foram realizados procedimentos de imunohistoquímica para GFAP em 5 animais dos grupos controle e DAGES de 30 dias e 5 animais dos grupos controle e DAGES de 90 dias. Foram capturadas imagens desses animais em 2 cortes a nível medial da substância negra para fazer contagem das células positivas para GFAP nessa região.

Foram realizadas também imunohistoquímica para PV em 3 animais dos grupos controle e DAGES de 90 dias para poder mensurar a quantidade e distribuição dos neurônios parvalbumina positiva na substância negra.

Os procedimentos imunohistoquímicos foram feito seguido o protocolo logo abaixo:

Os cortes foram retirados na solução crioprotetora, selecionados e logo após lavados em TF 0,1 M durante 10 minutos três vezes, foi feito uma recuperação antigênica em tampão citrato 0,01 M e pH 6.0 durante 04 horas em estufa (FANEM-Ltda.) a 60°C. Os cortes ao voltaram a ser lavados em TF 0,1 M durante 10 minutos três vezes e logo foi feito o bloqueio da peroxidase endógena com uma solução com 3% de peroxido de hidrogênio + 70% de metanol TF 0, 1M por 30 minutos. Assim os cortes foram pré-incubados em soro normal de cabra 10% por 30 minutos (solução preparada com TF 0,1 M + 0,3% de Triton).

Logo após os cortes foram incubados no anticorpo primário biotinilado: Para GFAP (anti-GFAP): diluição de 1:2000 (Sigma – Aldrich) ou para PV (anti – PV) em uma diluição de 1:500 (Sigma – Aldrich) com 1% de soro normal de cabra em TF 0,1 M + 0,3% de triton durante pelo menos 12 horas (overnight) na geladeira à temperatura de 4°C. Os 2 anticorpos primários foram feitos em mouse. Os cortes foram lavados em TF 0,1 M durante 10 minutos três vezes e incubados no anticorpo secundário: anticorpo biotinilado contra a espécie em que foi obtido o anticorpo primário (X - mouse) na concentração 1:200 em TF 0,1 M + 0,3% de

Triton durante 1,5 h à temperatura ambiente. Logo após os cortes foram lavados em TF 0,1 M e logo após incubados na solução de ABC (Avidina-Biotina-Peroxidase): Diluição 1:200 tanto para avidina quanto para Biotina em TF Triton 0,3% por 60 minutos à temperatura ambiente. A solução de ABC precisou ser feita com no mínimo 20 minutos antes para que a avidina e a biotina pudessem reagir. Novamente os cortes foram lavados em TF 0,1 M e foi feito com os cortes uma reação para evidenciar a peroxidase: As secções sagitais foram colocadas em peneiras imersas em 100 ml de TF 0,1 M contendo 25 mg de DAB (3,3' – Diamino Benzidina) diluído previamente em 1 ml de TF 0,1M e o tempo de reação de 20 minutos agitando os cortes em seguida foi acrescido 1 ml peróxido de hidrogênio por 10 minutos. Etapa realizada na capela de exaustão. Os cortes foram lavados em TF 0,1 M durante 10 minutos três vezes e montados em lâminas gelatinizadas e logo após secagem, os cortes passaram pelo processo de desidratação e logo e foram montados em Entelan. Foi utilizado o esmalte para fixação das lamínulas.

Captura de Imagens

As imagens das lâminas de Proteína Glial Fibrilar Ácida (GFAP) foram capturadas dos cortes de animais dos grupos controle e DAGES de 30 e 90 dias de 2º geração utilizando o microscópio Olympus (BX50 F – 3, Japão) acoplado a uma câmera de vídeo (Samsung, SHC41ONAD, Coréia). As imagens de Parvalbumina (PV) foram capturadas apenas dos cortes de animais dos grupos controle e DAGES de 90 dias de 2º geração.

Análise Morfológica (Fractal)

As imagens foram obtidas a partir de cortes de cérebro corados com DAB imunorreativas para GFAP. A partir dos dois cortes mais medial de uma serie de cérebro por animal (n=3-5), as imagens em 20 x de duas regiões da substância negra (=4 imagens/animal) foram tiradas com um microscópio Leica (DM 5500 – B acoplado a uma câmera de vídeo Leica (DFC 345FX). Imagens de vários planos z-focais foram tomadas cobrindo a espessura do tecido que mostra a coloração GFAP, a fim de capturar todas as projeções 3D de células no tecido. Utilizando o software ImageJ (NIH, EUA), as imagens binárias de projeção z-stack foram obtidos e as suas correspondentes saídas esqueletizadas foram analisados. Usando um gerador de seqüência (http://www.random.org/) oito células esqueletizadas por imagem (= 32 células / animal) foram aleatoriamente selecionados para análise fractal.

Quantificação da Análise Fractal

Análise fractal foi realizada através do plugin ImageJ FracLac (Karperien, A., FracLac para ImageJ, versão 2.5. Http://rsb.info.nih.gov/ij/plugins/fraclac/FLHelp/Introduction.htm. 1999-2012), utilizando o método de contagem de caixa. Este método sistematicamente cobre a imagem com 6 séries diferentemente posicionados de caixas de calibre decrescentes (45% da região de interesse (ROI) de 2 pixels) e registada a quantidade de caixas que continham a informação a partir da ROI. Assim, os valores médios para a dimensão fractal (Df = log N / log ε, N = contagem e ε = tamanho da caixa), lacunaridade (uma medida de heterogeneidade) e área de mandril (área do casco convexo, um polígono obtido ligando o mais longo projeções astrocíticos) foram obtidos a partir das 6 posições da grelha utilizados para cada célula selecionada aleatoriamente esqueletizada. As diferenças entre os grupos foram determinadas por um teste de soma de Mann-Whitney classificação usando o SyStat13, considerando-se como intervalo de confiança de 0,95.

Extração da substância negra e do corpo estriado

Para obtenção dos homogenados da substância negra (SN) e Estriado (ES), os animais (6 por grupo) foram anestesiados profundamente (via respiratória) com doses sub-letais do anestésico geral enflurano e decapitados. Os encéfalos foram rapidamente removidos em solução salina (NaCl 0.9%) gelada para dissecção da SN e ES de acordo com o protocolo mostrado nas figuras 1 e 2 respectivamente.

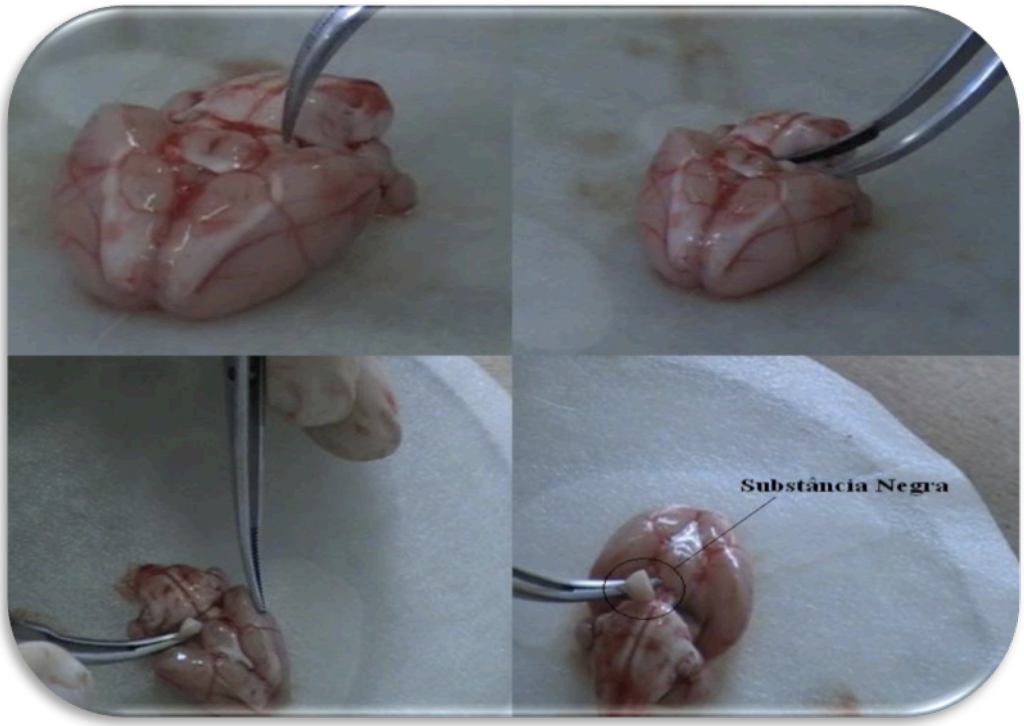

Figura 6. Extração de Substância Negra sobre superfície gelada em encéfalos recém - removidos. Duração do procedimento é de 1 a 2 minutos.

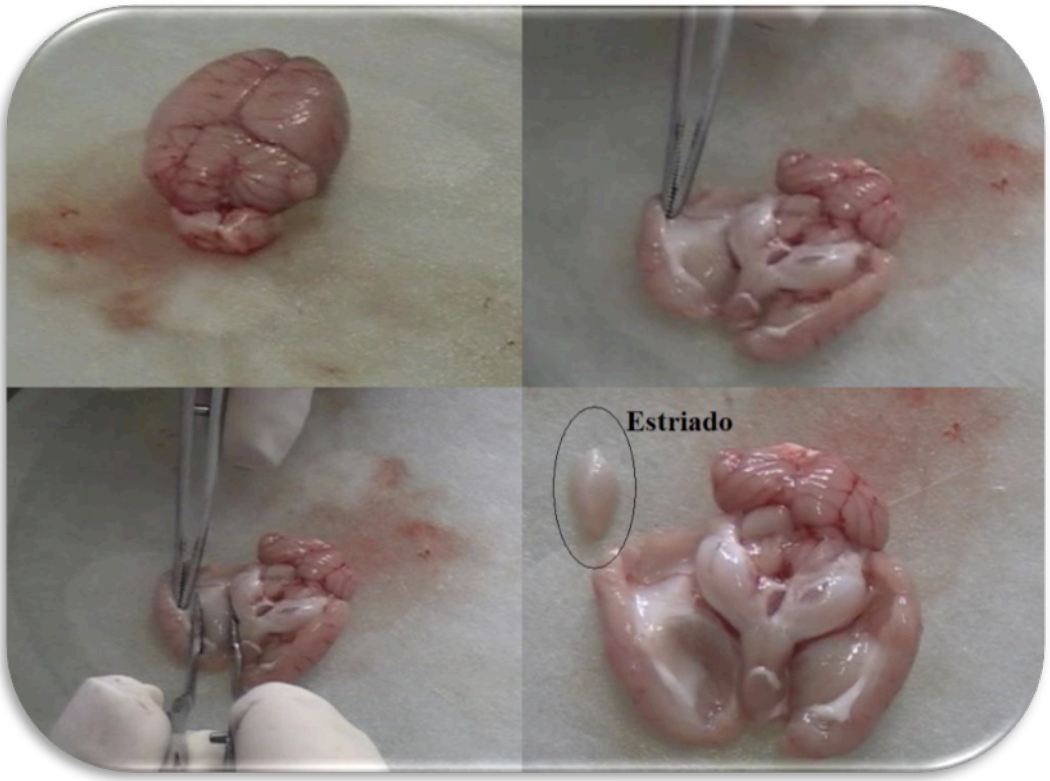

Figura 7. Extração do Estriado sobre superfície gelada em encéfalos recém-removidos. Duração do procedimento é de 1 a 2 minutos.

Ensaio para medir a atividade da enzima Glutamina Sintetase (GS)

A atividade enzimática da glutamina sintetase foi quantificada de acordo com Petito *et al., (1992)* utilizando seis animais por grupo. Utilizamos 100 µl de homogenados (pool de três animais) da Substância Negra (SN) e corpo estriado (ES). Adicionamos 200 µl da solução de mistura contendo em mM: 10 $MgCl_2$, 50 L-glutamato, 100 Imidazol-HC - pH 7,4; 10 β-mercaptoetanol, 50 Hidroxilamina-HCl, 10 de ATP. Esta solução foi incubada por 15 minutos a temperatura ambiente.

Para bloquear a reação utilizamos 800 µl de uma solução de bloqueio contendo em mM: 370 cloreto de ferro, 670 HCl e 200 de ácido tricloroacético. Após a centrifugação, utilizamos o sobrenadante para quantificar os níveis de γ – glutamil hidroxamato após a leitura no espectofotômetro em um cumprimento de onda de 530 ηm. Previamente foi realizada uma curva de γ – glutamil hidroxamato com concentrações variando de 50 a 500 µM. Todos os experimentos foram realizados em triplicatas e repetidos duas vezes. Antes da realização dos mesmos foram feitos experimentos piloto com animais alimentados com dieta Labina para melhor otimizar o protocolo.

Análise por Western Blotting

Os níveis protéicos de GFAP foram analisados pela técnica de *Western blotting*. Logo após a decapitação, os cérebros foram removidos e colocados em uma solução salina gelada. Cada região foi pesada na balança analítica (Marte BL320H) antes de ser homogeneizada no gelo dentro de 1 ml de solução contendo: Tris-Base – 20 mM/ $MgCl_2$ - 10 mM/ $CaCl_2$ - 0.6 mM/ EGTA- 0.5 mM/ DTT - 1 mM/ PMSF (-1 mM Aprotinina -5µg/ml/ Leupeptina - 2µg/ml/ Triton x-100 - 0.05%. A homogeneização foi feita para cada região contendo pool de 6 animais por grupo. Todo procedimento foi feito em gelo. Os tubos com o homogenato foram centrifugados por 30 minutos (12.000 r.p.m.) O sobrenadante foi recolhido e colocado em eppendorfs.

Para a corrida no gel, usamos amostras contendo 1.5 mg/ml. Preparamos amostras contendo 20 µl por poço. O tampão de amostra foi feito para um volume final de 16 ml contendo: 2 ml de Tris – HCl pH 6.8 com uma concentração de 0,5 M, 1,6 ml de glicerol, 3,2 ml de (Dodecil sulfato sódio) SDS 10% (peso/volume), 0,8 ml de 2 – mercaptoetanol e 0,4 ml de Brometofenol – azul.

Antes da amostra ser aplicada no gel a mesma foi aquecida juntamente com o tampão de amostra, finalizando com uma concentração (1.5 mg/ml). Essa mistura foi imersa em água fervente durante 3 minutos.

O gel de corrida foi preparado com 10% de acrilamida contendo 0.1% de SDS e utilizou-se 20 µl de cada amostra nos espaços demarcados pelo pente. Após a separação, as bandas de proteínas foram transferidas do gel para papel de nitrocelulose de acordo com Towbin (1979). A eletroforese durou 50 minutos numa voltagem de 150 V, sob uma corrente de 500 mA. Já a transferência para a membrana de nitrocelulose foi feita com uma voltagem de 60 V e uma corrente de 500 mA.

A membrana de nitrocelulose foi incubada em anticorpo policlonal feito em coelho anti-GFAP (Dako, 1:200) durante cerca de 12 horas. A membrana foi lavada em tampão fosfato-salina mais tween (TBS-T) por 10 mim e logo após foi colocado o anticorpo secundário biotinilado cabra contra coelho (Jackson, 1/1000) em tampão tris-salina mais tween (HST) por 1,5 h. Após lavagem de 10 min com HST as amostras foram incubadas no Kit ABC (10µl A + 10µB + 1980 µl de HST) por 1 h. Foram feitas lavagem sucessivas com TBS-T, HST, TBS-T na membrana e por fim foi utilizado Mix DAB líquido (Sigma Aldrich) para visualizar marcação colorimétrica das bandas. A normalização dos dados será feita em relação à marcação de beta-tubulina (Sigma, 1: 10.000) usada como padrão interno.

Tabela 4
Constituição dos géis de acrilamida

Reagentes	Lower gel	Upper gel
Água	2,5 ml	1,5 ml
Tampão Lower/ Upper	1,44 ml	600 µl
Acrilamida	2 ml	375 µl
SDS 10%	60 µl	24 µl
APS	80 µl	24 µl
TEMED	8 µl	4 µl

5. CAPÍTULO I

Astroglial reactivity in the nigro-striatal system is modified by essential fatty acid dietary restriction over two generations. (Manuscrito a ser submetido)

Astroglial reactivity in the nigro-striatal system is modified by essential fatty acid dietary restriction over two generations

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Running title: Unbalanced dietary levels of fatty acids induce astroglial enzymatic reactivity
Text pages: 25
Figures: 5
Tables: 4

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Introduction

It is well established that brain physiology during development and in adulthood depends of a wide range of astrocyte adaptive functions. Among these functions are metabolism of several neurotransmitters such as GABA and Glutamate (Bak *et al.*, 2006; Daikhin e Yudkoff, 2000), synthesis and secretion of trophic factors (Faissner *et al.*, 2010; Wiese et al., 2012), regulation of synapse formation and remodeling and control of synaptic strength coordinating activity among sets of neurons (Ullian et al., 2001; Pfrieger, 2002; Barker and Ullian, 2008; Diniz et al., 2012). Astroglia can also induce neurogenesis from adult neural stem cells (Song et al., 2002; Ashton et al., 2012).

Nevertheless, these glial cells are not a homogeneous population of cells. Astrocytes from distinct brain regions display unique phenotypic properties as well as functional heterogeneity (Wang and Bordey, 2008; Hewett, 2009). It has been discussed that such variety seems to be due to intrinsic and extrinsic mechanisms involving different progenitors and phenotypic plasticity regulated by signals from local neuronal populations (Hewett, 2009). Astrocytes in adjacent GABAergic nuclei of the basal ganglia, for example, can exhibit remarkable molecular heterogeneity in the expression of some conexins (Nagy et al. 1999) which play important roles in several aspects of intercellular communication and cell survival (Lin etal., 2003).

Beyond its importance under physiological conditions, astroglial activity is also fundamental in diverse types of brain insults (Parpura et al., 2012) and reduction in their metabolism is related to synaptic dysfunctions and neuronal vulnerability to lesions (ZOU *et al.*, 2010). Glial cells respond to traumatic and degenerative conditions of the central nervous system (CNS). This response is characterized by hyperplasia and hypertrophy of microglia and astrocytes (Strömberg et al., 1986; Maragakis and Rothstein, 2006). Usually astroglial activation can be accompanied by upregulation of the glial fibrillary acidic protein (GFAP), especially its phosphorylated isoform (Sullivan et al., 2012). Astrocytes play an important role in glutamate-induced excitotoxicity, clearing extracellular glutamate through the membrane transporters, and subsequently converting the incorporated glutamate into glutamine by the enzyme glutamine synthetase (GS). Some conditions of acute brain injury are also associated with the increased expression of GS in astrocytes as an indicator of reactivity (Lehmann et al., 2009).

Reactive astroglia synthesizes increased levels of bioactive substances which are related to their paracrine actions on neurons (Fawcett, 1994; Ridet et al., 1997) and can stimulate sprouting and neuroprotection (Schnell & Schwab,1993, Faulkner et al., 2004). In

the nigro-striatal system, it has been shown that astrocytes secrete a number of neurotrophic factors for dopaminergic neurons, including glial cell-line-derived neurotrophic factor (GDNF) (Lin et al., 1993), brain-derived neurotrophic factor (BDNF) (Knott et al., 2002) and mesencephalic astrocyte-derived neurotrophic factor (MANF) (Petrova et al., 2003). Astrocytes in the SN can also upregulate protease-activated receptor-1 (PAR-1) in Parkinson Disease which has been described as protective by increasing the activity of glutathione peroxidase (Ishida et al., 2006). Enriched environment induces astroglial reaction and protects the nigrostriatal dopaminergic system against oxidative effects of 6-OHDA (Anastasia *et al.*, 2009). Nevertheless, under some conditions, the astrocytic activation can occur even after dopaminergic cell death in the SN pars compacta (Kohutinicka *et al.*, 1998; Przedborski *et al.*, 2000) suggesting that overactivation of astrocytes may also play deleterious effects in this nucleus.

Neuroprotective actions of astrocytes also involve their ability to synthesize and release into the extracellular medium, essential fatty acids such as docosahexaenoic acid (DHA) and arachidonic acid (AA) which can exert important roles in the brain excitability and vascular responses (Vreugdenhil et al., 1996; Lauritzen et al., 2000; Blondeau et al., 2006). Under some pathological conditions such as brain ischemia and epilepsia, 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 that neuronal membrane phospholipids preferentially retain DHA but do not synthesize or release it readily (Moore et al., 2001). Therefore, DHA provided by astrocytes under physiological or pathological conditions contribute to modulate neuron–astrocyte cross-talk involved in several neuronal circuits (Strokin et al., 2003).

The importance of DHA on the structure and function of astrocytes has been demonstrated in some *in vitro* studies: Joardar et al. (2006), for example, using cerebral cortex astrocyte primary cultures, reported that DHA is involved in the astrocyte morphological differentiation induced by beta-adrenergic receptors. The same authors also showed that treatment of astrocyte cultures in medium containing low concentration of DHA impair their development, keeping them in an immature phenotype and modifying the expression of GFAP isoforms (Joardar & Das, 2007). DHA exerts protective effects on the viability of hamster cortical astrocytes (Champeil-Potokar et al., 2003) as well as promoting stimulatory effects on functional distribution of gap junctions in these cells (Champeil-Potokar et al., 2006). In vitro studies also showed that DHA modulates the activity of glutamate transporters

either in human embryonic kidney cell line (Berry et al., 2005) or in cortical astrocytes (Grintal et al, 2009).

Adopting a two-generation model of essential fatty acid (EFA) dietary restriction, we have recently shown that this type of nutritional insult induces signs of degeneration in SN dopaminergic and non-dopaminergic neurons. In addition, DHA depletion increases lipoperoxidation (LP) levels and reduces enzymatic anti-oxidant resource in the SN but not in the corpus striatum (CS) of young rats (Cardoso et al., 2012). In the present study, using the same experimental model, we investigated the repercussion of this EFA deficiency on functional and morphological aspects of astrocyte activity in the nigrostriatal system of young and adult animals. Considering that adequate levels of omega-3 fatty acids are crucial for differentiation and viability of neural cells, but that glial cells from distinct brain regions display distinct phenotypic plasticity, we hypothesized that chronic DHA depletion could induce a differential astrocytic reactivity in the SN and CS.

2. Materials and Methods

Adult female Wistar rats weighing 250–300 g were fed from mating throughout pregnancy and lactation on a control or an 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 and Lago (1973) and analyzed using gas chromatography. The control diet contained 5% of soybean oil with adequate amounts of saturated, monounsaturated, a-linolenic and linoleic fatty acids. The experimental diet contained 5% coconut oil with reduced levels of linoleic acid (~7 times reduction, corresponding to about 30% of recommended minimal dietary requirement for rodents (Bourre et al., 1990)) and α-linolenic acid (~12 times reduction), and higher levels of saturated (2 fold) and monounsaturated (2.5 fold) fatty acids. The diets were prepared at least twice a month and stored in a refrigerator at 4ºC.

The offspring (n = 108 males) of both groups were the object of the present study. At parturition, litter size, total litter weight and mean birth weight of the pups was recorded. Litters were then reduced to 6 pups each, by keeping the median weighted animals (identified with distinct marks). Dams and male pups were divided into two main groups according to the nutritional condition: control (C) and experimental rats (E). After weaning, on postnatal day (P) 21, the rat pups were separated and fed ad libitum the same diet as their respective mothers. They were the first generation of animals and were allowed to mate at 90–110 days to provide the second generation groups which were analyzed at 30–42 days (CF2Y and EF2Y groups) and at 90-110 days (CF2S and EF2A groups). In each group, animals 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 6:00 h). 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).

2.3. Protein extraction and Western blotting analysis

Ventral midbrain, cerebral cortex and corpus striatum homogenates containing samples of 6 animals per group were obtained at P35-42 from the CF2Y and EF2Y groups. Such samples were homogenized in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM MgCl2, 0,6 mM CaCl2, 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 protein content. An equal volume of sample buffer (62.5 mM Tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% mercaptoethanol and 0.002% bromophenol blue) was added, and samples were 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 nitrocellulose paper, as described by Towbin et al. (1979). The nitrocellulose blots were incubated with mouse anti-GFAP polyclonal antibody (1:500; Dako) or mouse anti-ß-actin polyclonal antibody (1:5000; Chemicon, Temecula, CA, USA) and then incubated for 3 h at room temperature. They were subsequently exposed to mouse secondary antibodies conjugated to horseradish peroxidase and developed with a 0.16% solution of 3- amino-9-ethylcarbazole in 50 nM sodium acetate buffer (pH 5) containing 0.05% Tween-20 and 0.03% H2O2. Digital images of the blots were obtained and the integrated optical density was estimated by using Labworks software (UVP Products, CA). GFAP protein levels were normalized to that of the b-actin protein that was used as an internal standard.

2.4. Glutamine synthetase activity

Each experimental day, three animals per group of young or adult animals were anesthetized with isofluorane and then decapitated. The regions containing the SN or CS were rapidly dissected and the pooled tissue of each group was homogenized in a 0.15 M KCl solution (1:5 w/v). The measurement of GS activity was performed according to Petito et al. (1992), with modifications. SN or CS 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 MgCl2 and 10 mM ATP and 10 mM 2-mercaptoethanol and incubated for 15 min 37°C. The reaction was stopped by adding 0.4 ml of a solution containing 370 mM $FeCl_3$, 200 mM TCA, 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 g-glutamyl hydroxamate. One unit was defined as the enzyme activity needed to form 1 mmole of g-glutamyl hydoroxamate per minute. At least two independent experiments were carried out in triplicate.

2.5. Immunohistochemical procedures

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% paraformaldeyde in 0.1 M phosphate buffer (PB), pH 7.4 (200 ml). Perfusion was always performed between 12:00 and 18:00, with a continuous infusion pump (Harvard equipment) through a cannula inserted into the left ventricle with a perfusion rate at 7.64 ml/min. After perfusion, the 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 solutions of 10, 20 and 30% sucrose in PB.

Brain blocks were serially cut on a freezing microtome (Leitz Wetzlar) into 50 mm-thick sections in the parasagittal plane, throughout the latero-medial extent of each hemi-brain. All sections were collected serially in PB and arranged in six series. The atlas of Paxinos and Watson (1986) was used to delimit the cytoarchitectonic regions. The series of sections used for immunohistochemistry was first treated with 0.01 M citrate buffer, pH 6.0, at 60°C for 2 h. Thereafter, free floating sections were rinsed in PB several times and incubated with mouse anti-GFAP monoclonal antibody (Sigma-Aldrich) diluted 1:2000 in PB containing 0.3% Triton X-100 (PBX) and 1% normal goat serum (NGS) 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 chromogen. Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. For the control of the staining specificity, some sections were subjected to an immunohistochemical procedure, omitting the primary antibody.

2.5. Quantitative analysis of the number of astrocytes at the middle level of Substantia nigra

In order to investigate if EFA dietary restriction could induce astrogliosis we calculated the number of astrocytes at the mid-level of the SN in five brains per group processed for GFAP immunohistochemistry. The quantitative analysis was performed in two parassagital sections at the middle level of SN, from the stereotaxic coordinate corresponding approximately to 1.8 mm lateral (plate nº 81) to 2.4 mm lateral (plate nº 82) from Paxinos and Watson, (1986). These sections were from one of 6 series obtained from the left side of the brain. A Leica DMLS microscope coupled to Samsung high level color camera (model SHC-410 NAD) was used to obtain digital images from immunoreacted brain sections (40 X objective plus 2 X magnification of the camera). Adjacent sampling windows throughout the extension of cytoarchitetonic limits of SN were analyzed using Image J 1.46 (NIH, USA) software to count all GFAP positive astrocytes in each section.

2.5.1 Morphological (fractal) analysis

Considering the heterogeneity of astrocytes and that when these cells become activated they undergo a series of morphological changes, we applied a fractal dimension (FD) analysis as a descriptive parameter for the surface complexity of these cells in the substantia nigra. Moreover, we also analyzed other morphometric parameters such as lacunarity and arborization area.

2.5.2 Image acquisition and astrocyte selection

Digital images were obtained from DAB stained brain slices immunoreacted for GFAP in 5 animals per EF2Y or CF2Y groups and in 3 animals per EF2A or CF2A groups. From one series containing 6 parasagittal brain sections, two of these sections, at the mid-level of SN, were analyzed per animal. Pictures from sampling two random areas per section of the substantia nigra (4 pictures/animal) were taken with a camera (Leica, DFC 345 FX) using Deconvolution microscopy (Leica, DM 5500-B). 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 were obtained and their corresponding skeletonized outputs were analyzed. Using a sequence generator (http://www.random.org/) eight skeletonized cells per image (= 32 cells/animal) were randomly selected for fractal analysis.

2.5.3 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 (45% of the region of interest (ROI) to 2 pixels) and registered the amount 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 arbor area (area of the convex Hull, a polygon obtained from connecting the longest astrocytic projections) were obtained from the 6 grid positions used for each randomly selected skeletonized cell. Differences between groups were determined by a Mann-Whitney Rank sum test using the SyStat13 for Windows, considering 0.95 as confidence interval.

3. Results

3.1 Body and Brain weights

Body weights of young and adult animals were significantly smaller in the EF2 groups as compared to the respective controls. Brain weights of young but not of adult animals were significantly smaller in the EF2 group, compared to control. Nevertheless, the brain weight /body weight ratio did not differ between the groups in each age (Table 3).

3.2 Glutamine synthetase activity in the SN and CS of young and adult animals

Figure 1 shows the results obtained in the SN and CS homogenates of young and adult animals of control and experimental groups. In the SN, no intergroup difference in the GS activity was observed in young animals (37.6±4.31 versus 42.8 ± 6.24 L-glutamyl hydroxamate/h/mg prot in the control) while a 2.5 fold increase was observed in the CS of the EF2Y group (58.31±14.71) compared to control (20.13±5.87, L-glutamyl hydroxamate/h/mg prot $p<0.01$). In EF2A animals, GS activity significantly increased in the SN and CS and the average values were, respectively, 4- and 2-fold higher than those of the control groups ($p < 0.001$).

Please, insert Figure 1 around here

3.3 GFAP isoforms expression in Western blot

Western blot analysis in homogenates of ventral midbrain, cerebral cortex and CS of young animals showed a different pattern of GFAP expression in the EF2Y group when compared to their respective controls (**Fig. 2)**. In the control condition this protein is expressed predominantly with 50 kDa, which corresponds to phosphorylated isoform. In the experimental condition, the expression of this isoform was reduced and the ~42 kDa non-phosphorylated isoform was more intensely labeled, especially in the ventral midbrain. In this later region, two additional isoforms were also visualized: one with molecular weight between 42 and 50 kDa and another with molecular weight smaller than 42 kDa.

Please, insert Figure 2 around here

3.4 Number of GFAP positive astrocytes at the middle level of SN in young and adult animals

Panel A in **Figure 3** shows low magnification images of representative sampling fields of parasagittal sections stained for GFAP through middle level of SN, comparing the GFAP immunoreactivity pattern in astrocytes of control and experimental groups in representative young and adult animals. Quantitative analysis carried out in 5 animals per group did not show differences between the groups either at young or adult stage although a tendency for fewer cells has been observed in most of EF2Y and EF2A animals when compared to their respective controls (**Fig. 4**). On average, the numbers of GFAP-immunoreactive cells in the mid-level of SN of young animals were 4918 ± 2055 and 3486 ± 1878 cells in control and experimental groups, respectively. These numbers in control and experimental adult animals were 6378 ± 1904 and 4870 ± 1539 cells, respectively.

Please, insert Figures 3 and 4 around here

3.5 Morphometric analysis of GFAP immunoreactive astrocytes in the Substantia Nigra

Panel A in **figure 5** shows z-stack projected images of representative sampling fields in parasagittal sections stained for GFAP through the mid-level of SN of control and experimental animals. Randomly selected skeletonized images obtained from the z-projected pictures were used in the analysis of descriptive parameters for astrocyte surface complexity. Panel B shows samples of these skeletonized images obtained in all groups analyzed. As can be seen, GFAP immunoreactive astrocyes show diverse forms, from small cells with short, thin and poorly ramified process to cells with longer, thin and more ramified processes, especially those of EF2Y group.

Please, insert Figure 5 around here

Quantification of morphometric analysis of fractal dimension, lacunarity and arborization area is illustrated in **Figure 6**. A significant increase in the FD ($p = 0.009$) and arborization area ($p = 0.004$) of astrocytes was observed in EF2Y group compared to its respective control while no intergroup difference was detected in the lacunarity ($p = 0.202$). In adult animals, no intergroup difference was detected for FD ($p = 0.594$) and lacunarity ($p = 0.878$). Although a significant increase was observed in the arborization area of EF2A group, compared to its respective control ($p = 0.005$).

Please, insert Figure 6 around here

4. Statistical analysis

Six animals from three litters per group were used each time. A total of 34 young and 54 adult animals were used. Biochemical data of glutamine synthetase enzymatic activity 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 Bouferroni as the post-hoc test. The analysis of body, brain weight and the number of GFAP positive cells was carried out using unpaired Student´s t-test and ANOVA followed by Bouferroni test to determine post hoc comparison among groups and ages.

5. Discussion

Essential fatty acid dietary restriction over two generations is able to induce oxidative stress in the SN but not in the CS of young animals (Cardoso et al., 2012). The present study investigated whether a differential astrocytic reaction could occur in the CS as a potential mechanism involved in its apparent resilience. Using morphological and functional parameters we also extended our analysis to adult animals of second generation in order to compare the repercussion of different levels of DHA depletion. The results partially corroborated our hypothesis, demonstrating that an increase in the GS activity was detected in the CS of experimental young animals. However, in adult animals fed the deficient diet, the functional activity of this enzyme was greater in both SN and CS, compared to control group. Intriguing and adverse effects were also observed in morphological reactivity of SN astrocytes either in young or adult animals submitted to this type of nutritional insult.

Under physiological conditions, GS is mainly expressed in astrocytes and play important roles in the glutamate and ammonia metabolism. Their activity is usually stimulated by elevated glutamate concentrations in the extracellular environment (Zou et al., 2010) but can also be triggered under deleterious conditions when glutamate uptake by astrocytes is reduced (Feoli et al., 2008). The distribution of GS in several regions of the rat central nervous system was described by Norenberg (1979) using an immunohistochemical approach. According to these authors, the intensity of astrocyte GS staining varied greatly in different regions and correlated well with sites of higher glutamatergic activity. In their comparative analysis, higher levels of GS staining were noted in the CS when compared to SN. Our results in young animals fed the experimental diet, seems to be coherent with the hypothesis raised in this previous report indicating a higher enzymatic reactivity in astrocytes of the CS. Such findings are also in concordance with a greater superoxide dismutase (SOD) enzymatic

activity in the CS of young animals reported by Cardoso et al., (2012) using the same experimental model herein adopted. It has been demonstrated that the level of extracellular - SOD mRNA is significantly greater in astrocytes than in neurons and microglia and that SOD activity in astrocytes contribute to the defensive mechanism against oxidative stress in brain (Litsuka et al., 2012). Growing evidence also indicates the importance of astrocytes against oxidative stress-mediated neuronal death (Takuma et al., 2004). Astrocytes are also involved in the glutathione synthesis and provide glutathione precursors to neurons (Gringen et al., 2000). Thus, it is possible that in young animals, enzymatic astrocytic reaction in the CS contributed to resilience of this nucleus to oxidative insult induced by DHA deficiency.

The lack of intergroup difference in the GS activity observed in the SN of young animals was accompanied by an increase in some indices of morphological reactivity such as fractal dimension and arborization area of astrocytes in the EF2Y group. 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 and Mucke, 1993). In the present study we observed that fractal dimension modifications occurred in young but not in adult experimental animals and some points should be considered in the discussion of this data. Alterations in the fatty acid composition of astrocytes induced by a multi-generation model of omega-3 fatty acid dietary restriction were described by Bourre et al., (1984). According to these authors, DHA depletion in astrocyte membrane phopholipids was higher in young (6 fold) than in adult animals (3 fold) in contrast to observations in neurons, where this type of depletion was more expressive in adult rats. The loss of dopaminergic neurons in the SN of EF2Y animals 30-42 days old was ~20 % in conditions of 50% DHA depletion in the midbrain (Passos et al., 2012). Recent evidence in our lab has indicated that this neuronal loss is about 50% in adult animals when DHA levels was reduced ~65% in the SN (Cardoso et al., 2012, unpublished results). Therefore, it is possible to speculate that the morphological changes herein observed in SN astrocytes of young animals could be due to a potential compensatory and reactive mechanism triggered in these cells in conditions of a higher DHA depletion in their membranes and not only related to the magnitude of dopaminergic cell loss.

The modifications observed in fractal dimension and arborization area of astrocytes in EF2Y animals occurred in the absence of proliferation of these cells, considering the average number of astrocytes herein estimated at the mid-level of SN. Inexistence of astrocyte proliferation was also observed in our experimental adult animals. A number of studies have shown that consistent 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 lack of apparent modifications in the lacunarity index in the SN, either in young or adult animals fed the experimental diet, seems to be consistent with the lack of astrocyte proliferation in the SN. Astrocyte proliferation in the SN as a consequence of dopaminergic cell loss is a matter of discussion in the literature. Although some experimental models of Parkinson disease (PD) in rodents 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 (see reviews in Teissman et al., 2003; McGeer and McGeer, 2008). Interestingly, a study on humans demonstrated surprisingly low reactive astrocytosis in the SN of PD (Mirza et al., 2000).

Another parameter used in the present study to investigate astrocytic reaction in conditions of DHA depletion was the expression of different isoforms of GFAP. It has been described that some conditions of brain insult, including those involving increased levels of oxidative stress are able to stimulate the expression of the phosphorylated isoform of GFAP (Sullivan et al., 2012). On the other hand, using cerebral cortex astrocyte primary cultures and coconut oil as the source of fatty acids to the medium, Joardar et al., (2007) reported that DHA deficiency in the culture medium modified the expression of GFAP isoforms and induced an immature phenotype in these astrocytes. Our findings in EF2 young animals demonstrated that in the SN, CS as well as in the cerebral cortex, the expression of the 50 kDa phosphorilated isoform was reduced while a 42 kDa isoform was increased. In the SN, two additional isoforms were also detected, one with molecular weight between 42 - 50 kDa and another with molecular weight smaller than 42 kDa. Such modifications herein detected in vivo, seems to reinforce the influence of low DHA levels in modifying the expression of GFAP isoforms detected by Joardar et al., (2007) in vitro. However, our results in young animals demonstrated that even under DHA depletion in the midbrain, SN astrocytes were able to develop phenotypic changes indicative of reactive differentiation, considering the greater indices of fractal dimension and arborization area.

Modifications in the GFAP expression characterized by an increase in the non-phosphorilated 42 kDa isoform without reduction in the 50 kDa isoform, was reported by Grintal et al. (2009), treating cerebral cortex astrocyte cultures with medium supplemented with several trophic factors. In that situation, astrocytic reactivity was present and characterized by elevated GS activity among other features involved in the glutamate uptake. It has been shown that high levels of extracellular glutamate and intracellular calcium stimulate phosphorylation of GFAP (Rodnight et al., 1997). Preliminary results of our lab

obtained in the SN homogenates of EF2A animals have indicated that despite a greater expression of 42 kDa GFAP isoform, compared to that observed in the control group, the reduction in the 50 KDa phosphorilated isoform was not so intense in this group as detected in young animals. These experiments should be complemented. Nevertheless, if future experiments confirm this preliminary data, it is possible that the 4-fold increase in the GS activity observed in the SN of EF2A group, concomitant with modifications in GFAP expression, could be partially due to potential increase in the glutamatergic activity into this nucleus. It has been shown that overactivation of glutamatergic neuronal afferents to SN causes excitotoxicity-induced neurodegeneration in this nucleus (Gonzalez-Hernandez et al., 1997). It is worth noting that in EF2 adult animals, higher levels of oxidative stress were observed both in the SN and CS compared to their respective controls (Cardoso et al., 2013; unpublished results). These authors also detected higher levels of nitric oxide in the CS and fewer number of BDNF positive cells in the SN of animals fed with the EFA deficient diet. Thus, the intense GS activity observed in the SN of our experimental adult animals could be occurring as a consequence of multifactorial effects inducing progressive neuronal degeneration in this nucleus.

In conclusion the present findings demonstrate for the first time that EFA dietary restriction over two generations is able to induce adverse effects on morpho-functional astrocyte reactivity in nuclei of basal ganglia. Enzymatic reaction in the CS of young animals reinforces our initial hypothesis that these cells could contribute to CS resilience to oxidative insult induced by DHA depletion. On the other hand, in adult animals, this functional response occurred in both SN and CS and was independent of morphological changes and proliferation of these cells. Taken together, the results reinforce previous evidence that under pathological or degenerative conditions a differential phenotypic plasticity in astrocytes can occurs among brain regions even in those functionally related.

Conflict of interest

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

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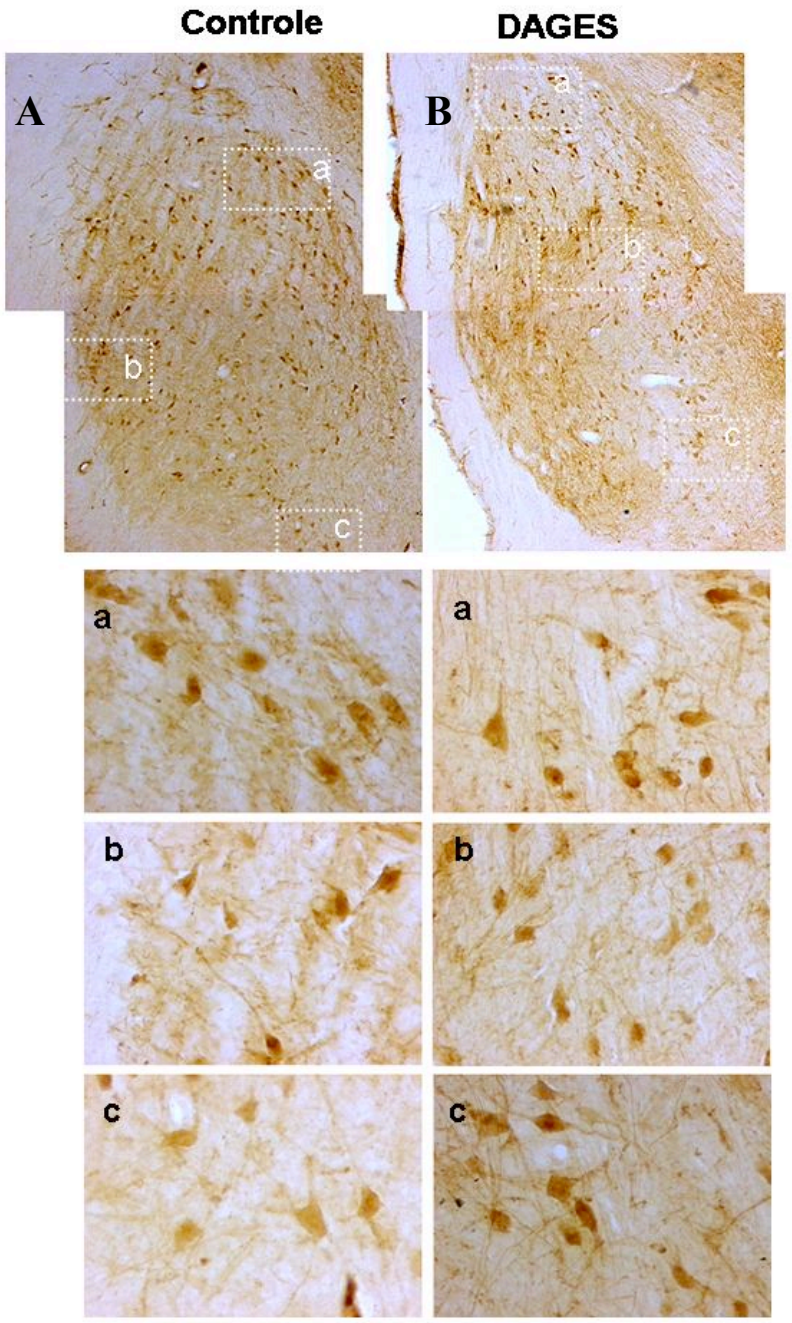
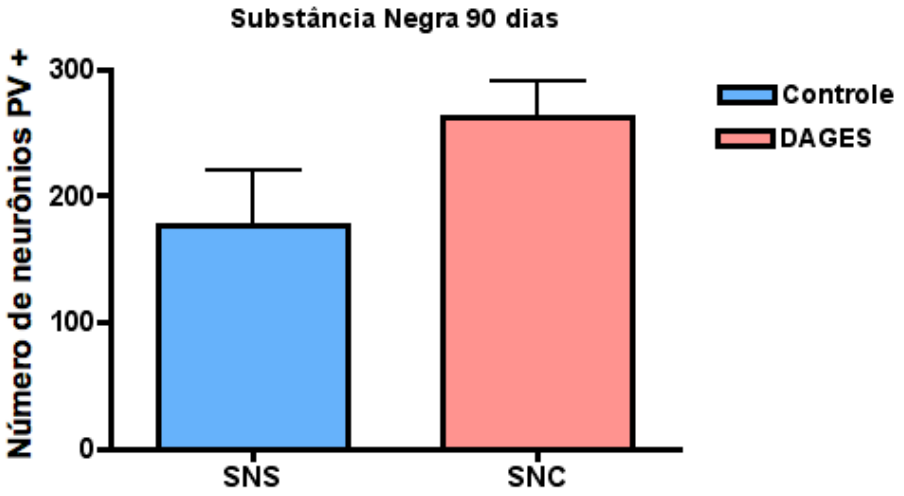
6. RESULTADO COMPLEMENTAR

Distribuição de neurônios GABAérgicos Imunorreativos á parvalbumina na SN.



Imagens representativas de secções parassagitais do encéfalo imunorreagidas para Parvalbumina ao nível medial da substantia nigra (SN) de animais adultos da segunda geração alimentados com dieta controle ou experimental. Imagens em baixa magnificação dos grupos controle (A) e experimental (B) mostram a distribuição de células contendo parvalbumina nos limites citoarquitetônicos da SN. Imagens em maior magnificação de (a), (b) and (c) mostram características fenotípicas de células parvalbumina-positivas em diferentes regiões da SN de ambos os grupos.

Análise preliminar quantitativa de neurônios GABAérgicos que contém parvalbumina na SN dos grupos controle e experimental.



Na substância negra existem neurônios GABAérgicos os quais podem ser caracterizados pela presença da proteína moduladora de cálcio, Parvalbumina.

Análise quantitativa preliminar realizada na SN de 4 animais do grupo DAGES e 3 animais do grupo controle mostraram que o número de células que contém parvalbumina no grupo DAGES não difere do respectivo grupo controle. Mesmo considerando que essa análise ainda precisa ser complementada com um número maior de animais não evidenciamos até o presente momento qualquer tendência a redução do número de células parvalbumina positivas no grupo experimental.

Se futuros experimentos confirmarem os nossos resultados preliminares, devemos considerar a possibilidade de que esses neurônios podem ser preservados diante do insulto oxidativo na SN. Isso não quer dizer que essa restrição dietética não seja capaz de diminuir o número de neurônios GABAérgicos. Evidências em pacientes parkinsonianos têm demostrado que a parvalbumina como proteína moduladora de cálcio podem passar a ser expressa em subpopulação de neurônios não GABAérgicos da SN em condicões de injúrias excitotóxicas e de estresse oxidativo (SOÓS J, 2004).

Assim é possível que mesmo diante da restrição dietética em AGEs, subpopulações neunonais da SN podem apresentar respostas plásticas que conferem aos mesmos uma maior resistência.

7. CONSIDERAÇÕES FINAIS

Investigamos no presente trabalho se uma restrição dietética em ácidos graxos essenciais por duas gerações é capaz de induzir alterações morfofuncionais nos astrócitos e diminuição do número de neurônios GABAérgicos nigrais de ratos jovens e/ou adultos.

Evidências obtidas em nosso laboratório primeiramente mostraram que animais jovens submetidos a esse modelo de restrição dietética por duas gerações apresentam um menor número de neurônios dopaminérgicos na SN (PASSOS *et al.*, 2012). Mais recentemente, foi também evidenciado que um dos potenciais mecanismos envolvidos com a perda de células dopaminérgicas deve-se a um aumentado nível de peroxidação lipídica associado a uma diminuição na capacidade antioxidante SN mais não do CE (CARDOSO *et al.*, 2012).

Por isso resolvemos investigar potenciais mecanismos envolvidos com atividade das células da glia, especificamente dos astrócitos com o objetivo de analisar se os mesmos contribuem com a resiliência do CE previamente observada em animais jovens e ampliamos o nosso estudos para analisar animais adultos submetidos a restrição em AGEs. Como parâmetro metabólico, avalíamos a atividade da enzima Glutamina Sintetase (GS).

Nossos resultados mostraram que a atividade da glutamina sintetase está aumentada nos animais DAGES de 30 dias apenas no CE, mas não na SN e isso com um nível de depleção de DHA de ≈ 50%.

Já nos animais adultos quando o nível de depleção em DHA é de ≈ 65% a atividade da GS aumenta muito na SN dos animais do grupo DAGES (≈ 4 vezes maior) em comparação aos animais controle.

Essa enzima é encontrada de forma diferente de acordo com a região do cérebro e provavelmente está relacionada com a atividade sináptica glutamatérgica da região. Nosso resultado mostrou uma maior atividade na enzima no CE o que está de acordo com os estudos de Norenberg (1979) que encontra uma maior quantidade de marcação para a GS no CE em relação a SN.

Nos animais adultos a depleção de DHA aumenta associado a um aumento na perda de células dopaminérgicas. Nessa situação observamos um aumento da atividade na GS na SN de animais experimentais, esse resultado mostra que o nível de DHA interfere na metabolização do glutamato. É possíveis que os efeitos deletérios desencadeados por esse modelo experimental envolva excitotoxicidade induzida por um potencial aumento nos níveis de glutamato.

A maturação celular de astrócitos pode também ser avaliada e pela fosforilação do GFAP, pois um aumento da expressão da banda fosforilada de 50 KDa está relacionada com uma maior reatividade. Como parâmetro para analise de reatividade astrocitária avaliamos se restrição dietética induz alteração na expressão da proteína GFAP, utilizando a técnica de imuno blot.

Os nossos resultados mostraram que tanto na SN, CE e cortex cerebral de animais jovens de 30 dias, houve uma menor expressão na isoforma fosforilada (50 KDa) e um aumento da isoforma não fosforilada (42 KDa). A diferença é que na SN foram expressas duas isoformas adicionais: uma com peso molecular entre 42 e 50 KDa e uma outra com peso molecular menor do que 42 KDa.

Já é bem evidenciado na literatura que em condições de isquemia ou de um insulto a glia reage do ponto de vista morfológico, observa-se um aumento na expressão da banda fosforilada de GFAP (SULLIVAN *et al.*, 2012). Por outro lado, um estudo *in vitro*, mostrou que ao cultivar culturas corticais de astrócitos em um meio que induz a reatividade, os níveis da isoforma não fosforilada de GFAP aumenta enquanto que os da isoforma fosforilada não diminuiu (GRINTAL *et al.*, 2009).

O nosso resultado *in vivo* foi similar ao obtido por Joardar *et al.,* (2007) cultivando astrócitos corticais com óleo de coco deficiente em DHA o que induziu a expressão de várias isoformas com pesos moleculares mais baixos que 50 KDa.

Avalíamos o número de células GFAP positivas na SN de animais 30 e 90 dias e não percebemos uma proliferação celular tanto nos animais jovens quantos nos animais adultos. Esse dado não está de acordo com alguns estudos na literatura que mostra que após lesão em neurônios dopaminérgicos na SN há um aumento no número de células imunorreativas para GFAP (RODRIGUES *et al.*, 2003). Contudo, um estudo em cérebros post mortem de pacientes parkinsonianos mostrou a inexistência de proliferação de astrócitos na SN (MIRZA *et al.*, 2000).

Em modelo de restrição dietética 65% de DHA foi visto que em animais adultos não houve proliferação de células imunorreativas para GFAP na área CA1 do hipocampo (LATOUR *et al.*, 2013). No entanto, não há evidência na literatura de uma perda neuronal nesta região como consequência da restrição em AGEs (AHMAD *et al.*, 2002).

Utilizamos a dimensão fractal para avaliar a reatividade morfológica dos astrócitos. Nossos resultados mostraram que houve um aumento na dimensão fractal nos animais DAGES de 30 dias e um aumentada área de arborização tantos nos animais de jovens quanto nos animais adultos.

Os resultados demostram que aos 30 dias os prolongamentos dos astrócitos estão aumentados, mesmo sem aumento no número dessas células.

Em conjunto o nossos resultados corroboram a nossa hipótese inicial que uma restrição dietética em ácidos graxos essenciais pode induzir alterações nas células da glia do sistema nigroestriatal. Evidências obtidas na literatura relatam que diante de lesões neuronais na substância negra, o número de astrócitos aumenta (RODRIGUES, GOMIDE *et al.*, 2003).

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9. ANEXOS

Tabelas e figuras do artigo do capitulo I

Artigos em Colaboração

Parecer do Comitê de Ética

9.1 Figuras e tabelas do artigo (Capitulo I)

TABELAS

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[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

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

Fatty acids	Control diet	Experimental 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. Bold values indicate $p < 0.001$.

Table 3 Body and Brain weights

	Body weight	Brain weight	Brain weight/body weight ratio
CF2 Y	79.65 ± 14.87 ; (*n* = 33)	1.69 ± 0,06; (n=08)	0,021 ± 0,004; (n=08)
EF2 Y	71.91±10.09*; (*n* = 40)	1,48 ± 0,123 ***;(n=10)	0,020 ± 0,010; (n=10)
CF2 A	385,46 ± 41,75; (n=15)	2,05 ± 0,18; (n=06)	0,0050 ± 0,004 ; (n=06)
EF2 A	338,28 ± 36,68 **; (n=2	1,91 ± 0,12; (n=09)	0,0056 ± 0,005 ; (n=09)

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

FIGURAS

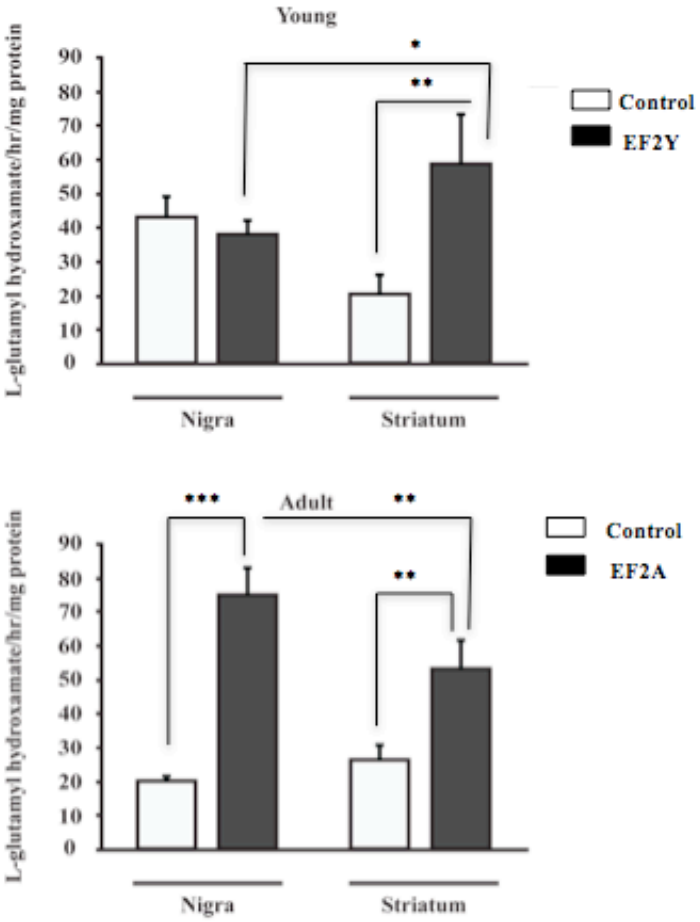

Figure 1. Quantification of glutamine synthetase activity.
Graphs show glutamine synthetase activity in substantia nigra (Nigra) and corpus striatum (Striatum)homogenates of second generation young (upper graph) and adult (bottom graph) rats fed control (white bars) and essential fatty acid deficient (EF2Y and EF2A, grey bars) diets. Each bar represents the mean ± SD obtained from three independent experiments . *Indicates a signifcant diference (*P<0.05; **P<0.01; ***P<0.001 ANOVA, followed by the Bouferroni test) between groups and regions.

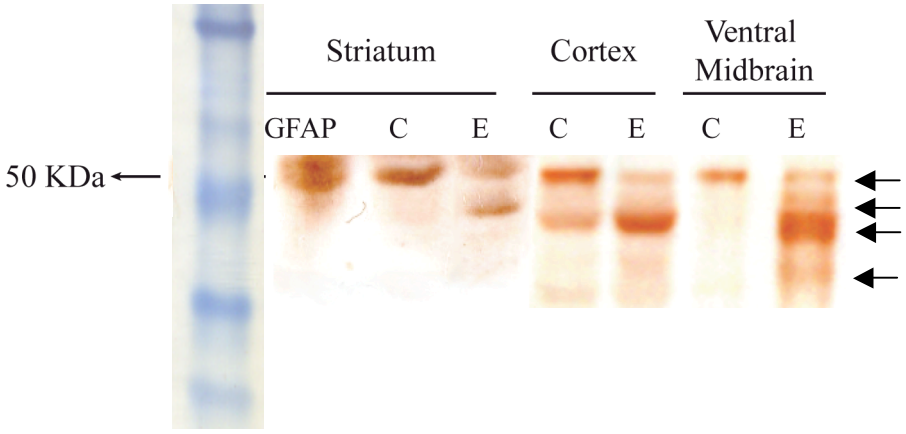

Figure 2. Effect of EFA dietary restriction over two generations on GFAP protein isoform expression in the corpus striatum, cerebral cortex and ventral midbrain of young animals. Note the reduction in GFAP-immunoreactive band of 50 kDa and the greater expression of ~42 kDa isoform in homogenates of experimental animals (E) compared to control (C). In the ventral midbrain, two additional bands were also detected in animals fed the essential fatty acid deficient (E) diet. Molecular weight marker and purified GFAP were used as control (GFAP lane).

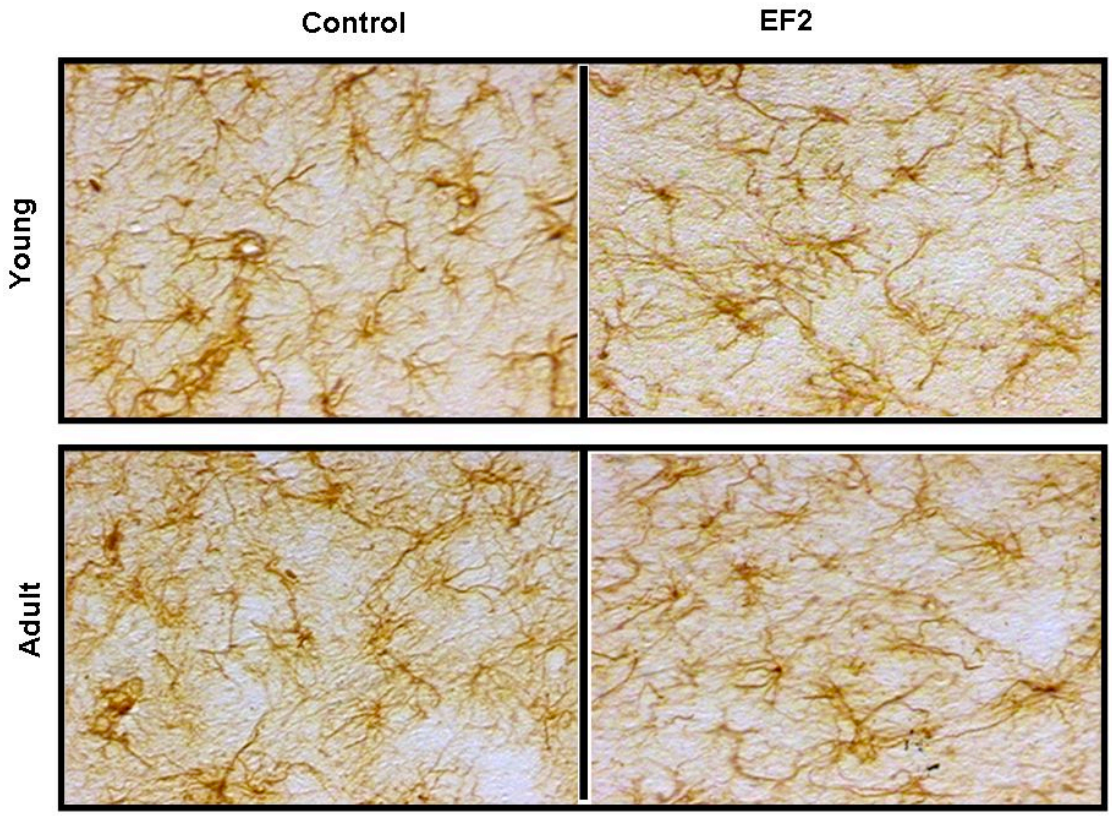

Figure 3. Low magnification images showing GFAP immunoreactivity pattern in astrocytes located at middle level of SN in representative young and adult animals of control and EF2 groups.

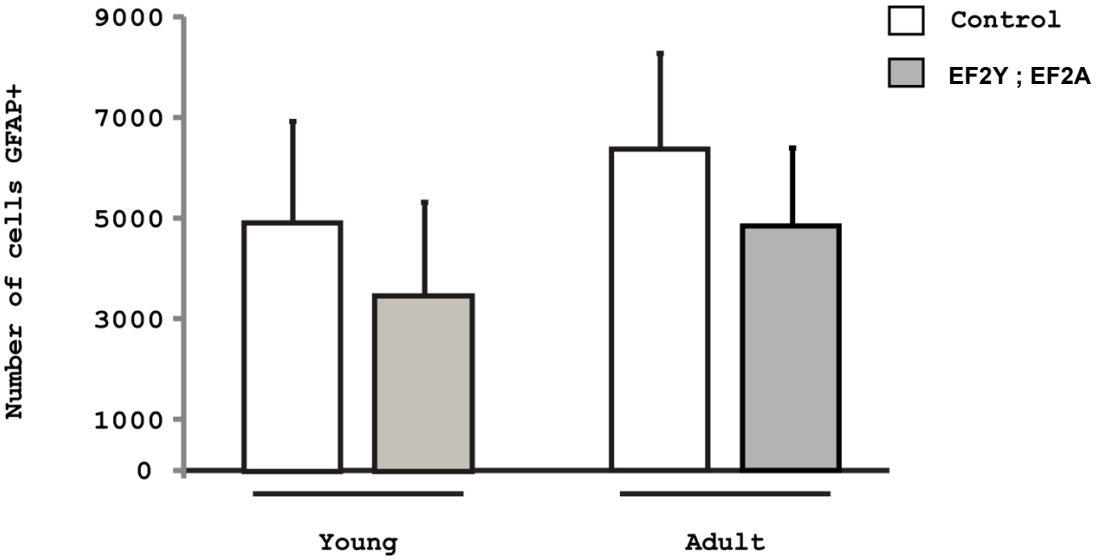

Figure 4. Quantification of GFAP positive cells in the substantia nigra.
Graph shows average number of GFAP immunoreactive astrocytes in two parasaagittal sections at the middle level of substantia nigra in second generation young and adult rats (n=5 per group) fed control (white bars) and essential fatty acid deficient (grey bars) diets. Error bars show standard deviation. No significant difference between the groups was detected.

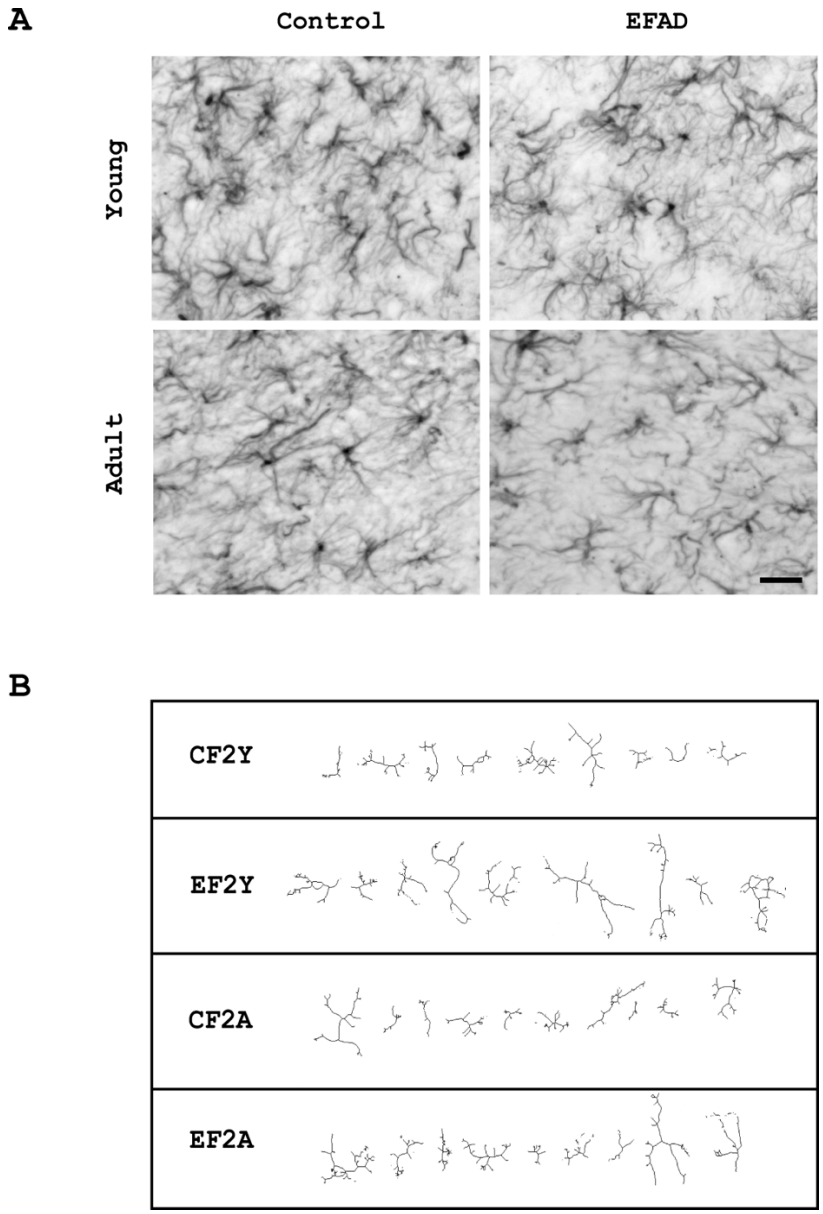

Figure 5. Representative z-stack projections and skeletonized cells used for fractal analysis.

(A) Representative projections of z-stack pictures taken from regions of susbtantia nigra immunoreactive for GFAP from second generation young and adult rats fed control or essential fatty acid deficient (EFAD) diets. Scale bar 50µm. **(B)** Representative skeletonized cells obtained from z-stack projections as in A, used for box counting fractal analysis. C = control diet; E = essential fatty acid deficient diet; F2 = second generation; Y = young; A = adult.

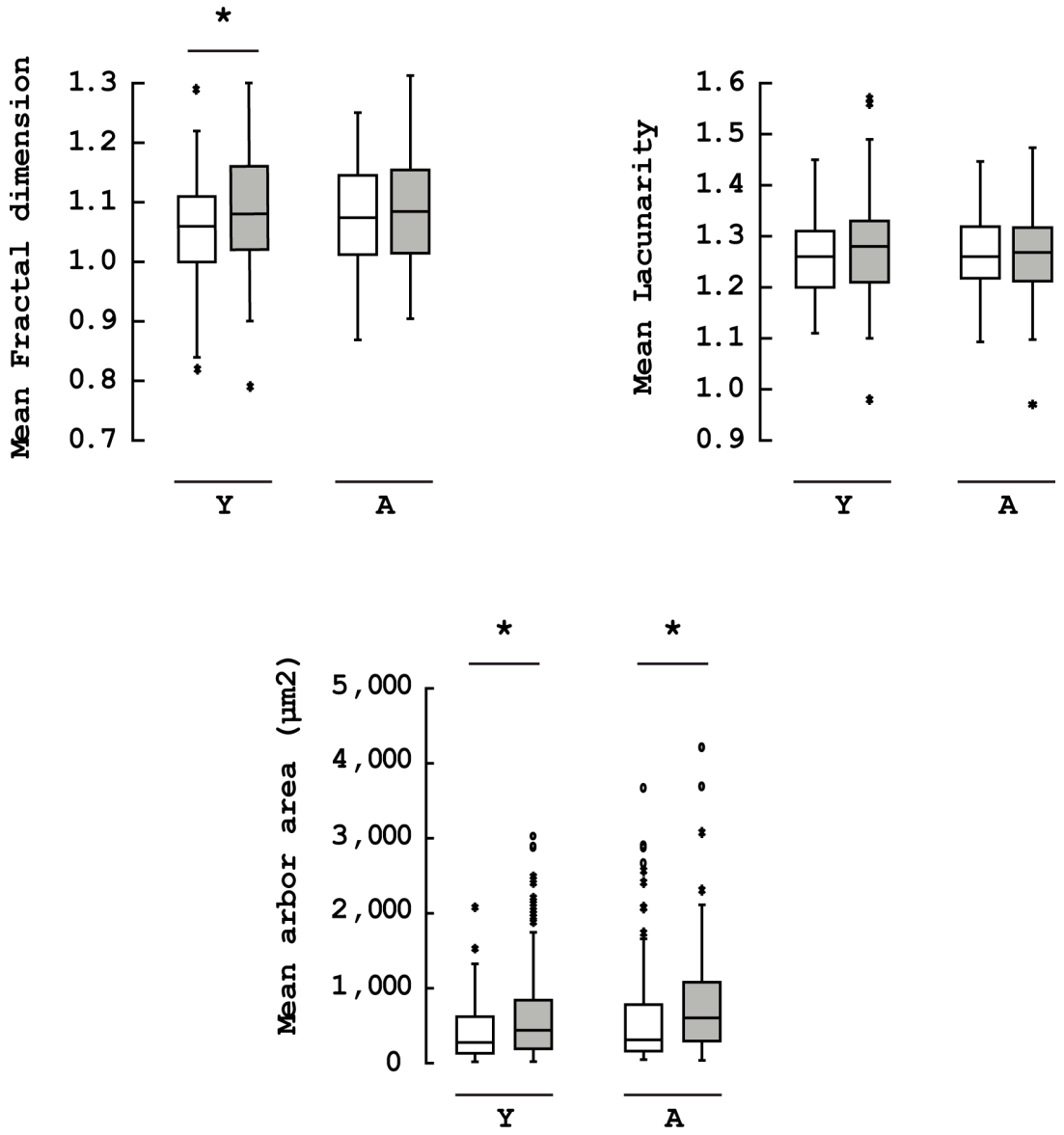

Figure. 6. Fractal dimension, lacunarity and arbor area of astrocytes.
Box plots show mean distribution values (see fractal analysis methods) of fractal dimension, lacunarity and arbor area of astrocytes from second generation young (Y) and adult (A) rats fed control (white boxes) and essential fatty acid deficient (EF2Y and EF2A, grey boxes) groups. On the box plot, the line indicates the median, the box represents the 25th and 75th percentile, and bars show minimum and maximum values. Circles on the plot area represent outliers. * $P < 0.01$ compared to control group (Mann-Whitney Rank sum test).

9.2 Artigos em Colaboração

9.2.1 Artigo científico

Differential vulnerability of substantia nigra and corpus striatum to oxidative insult Induced by reduced dietary levels of essential fatty acids. (publicado em Frontiers in Human Neuroscience, Volume 6, artigo 249, pag. 1 – 10; 2012.

9.2.2 Manuscrito do artigo científico:

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Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids

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Oxidative stress (OS) has been implicated in the etiology of certain neurodegenerative disorders. Some of these disorders have been associated with unbalanced levels of essential fatty acids (EFA). The response of certain brain regions to OS, however, is not uniform and a selective vulnerability or resilience can occur. In our previous study on rat brains, we observed that a two-generation EFA dietary restriction reduced the number and size of dopaminergic neurons in the substantia nigra (SN) rostro-dorso-medial. To understand whether OS contributes to this effect, we assessed the status of lipid peroxidation (LP) and anti-oxidant markers in both SN and corpus striatum (CS) of rats submitted to this dietary treatment for one (F1) or two (F2) generations. Wistar rats were raised from conception on control or experimental diets containing adequate or reduced levels of linoleic and α-linolenic fatty acids, respectively. LP was measured using the thiobarbituric acid reaction method (TBARS) and the total superoxide dismutase (t-SOD) and catalase (CAT) enzymatic activities were assessed. The experimental diet significantly reduced the docosahexaenoic acid (DHA) levels of SN phospholipids in the F1 (~28%) and F2 (~50%) groups. In F1 adult animals of the experimental group there was no LP in both SN and CS. Consistently, there was a significant increase in the t-SOD activity ($p < 0.01$) in both regions. In EF2 young animals, degeneration in dopaminergic and non-dopaminergic neurons and a significant increase in LP ($p < 0.01$) and decrease in the CAT activity ($p < 0.001$) were detected in the SN, while no inter-group difference was found for these parameters in the CS. Conversely, a significant increase in t-SOD activity ($p < 0.05$) was detected in the CS of the experimental group compared to the control. The results show that unbalanced EFA dietary levels reduce the redox balance in the SN and reveal mechanisms of resilience in the CS under this stressful condition.

Keywords: substantia nigra, corpus striatum, oxidative stress, superoxide dismutase, catalase, lipid peroxidation, DHA, neurodegeneration

INTRODUCTION

Docosahexaenoic acid (DHA) and arachidonic acid (AA) are long chain polyunsaturated fatty acids (LC-PUFA) which play important roles as critical modulators of brain function under physiological or pathological conditions (Zhang et al., 2011). They are derived from the essential fatty acids (EFA) α-linolenic and linoleic acids, respectively, and can exert opposite effects on brain metabolism (Schmitz and Ecker, 2008). Imbalance in their levels, early in life, and especially DHA deficiency, can decrease anti-inflammatory responses that can induce neurodegeneration (Yavin, 2006; Schmitz and Ecker, 2008). Recent studies using microarray technology have shown that DHA is able to regulate the transcription of many genes related to oxidative stress (OS), cell signaling, and apoptosis (Kitajka et al., 2004; Lapillonne et al.,

2004; Yavin, 2006). Consistent with this evidence, it has been demonstrated that DHA protects against peroxidative damage of lipids and proteins in developing and adult brains in experimental models of ischemia-reperfusion (Glozman et al., 1998; Green et al., 2001; Pan et al., 2009; Mayurasakorn et al., 2011) or reduce OS-induced apoptosis of retina photoreceptors (Rotstein et al., 2003). Moreover, the DHA-derived docosanoid, named neuroprotectin D1, protects human retinal pigment epithelial cells from OS (Mukherjee et al., 2004) as well as inhibits brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression (Marcheselli et al., 2003).

It is well established that OS is caused by the disequilibrium between the production and detoxification of highly reactive oxygen species (ROS), including singlet oxygen, superoxide anion, and hydrogen peroxide, which can disrupt the redox balance inside cells if not properly neutralized. The superoxide anion is known to induce protein and nucleic acid dysfunction and to initiate lipid peroxidation (LP) (Kohen and Nyska, 2002). Endogenous anti-oxidant mechanisms against superoxides include a series of linked enzyme reactions. The first of these enzymes is superoxide dismutase (SOD; EC1.15.1.1), that converts superoxide anion to hydrogen peroxide (H_2O_2), which can be removed by catalase (CAT; EC 1.11.1.6) and/or glutathione peroxidase (GPx; EC 1.11.1.9) (Kohen and Nyska, 2002; Melo et al., 2011).

Neuron response to OS is not uniform in the brain. This differential vulnerability depends on a number of factors including high intrinsic OS, high demand for ROS-based intracellular signaling, low ATP production, mitochondrial dysfunction, and high inflammatory response (Wang and Michaelis, 2010). Strong evidence indicates that OS may be one of the most important mechanisms involved in the etiology and evolution of a number of neurodegenerative diseases (Hashimoto and Hossain, 2011; Thomas and Beal, 2007; Melo et al., 2011). DHA is considered as a potential target for therapeutic intervention in some of these disorders, including Parkinson's Disease (PD), where the dopaminergic neurons of substantia nigra (SN) are especially affected by OS and mitochondrial dysfunction (Jenner et al., 1992; Sayre et al., 2001). In experimental models of PD, for example, it has been 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 OS (Bousquet et al., 2008; Cansev et al., 2008). Moreover, DHA supplementation is able to increase the SOD activity in the corpus striatum (CS) (Sarsilmaz et al., 2003) as well as significantly decrease cyclooxigenase-2 activity and prostaglandin E2 levels in the SN, decreasing MPTP-induced dopaminergic cell death (Ozsoy et al., 2011). Conversely, combination of successive parity and α-linolenic acid deficient maternal diet reduced the number of dopaminergic neurons in the rat SN pars compacta and ventral tegmental area of adult offspring (Ahmad et al., 2008).

Recent evidence from our laboratory, adopting a two generation model of EFA dietary restriction and stereological assessment, showed a differential vulnerability of two distinct SN dopaminergic cell populations to this type of nutritional insult. In addition to a reduction in the number of dopaminergic neurons in the SN rostro-dorso-medial region, this dietary treatment was able to change body and brain weights, TH protein levels, and the size of these neurons in young animals (Passos et al., 2012). The mechanisms involved in such effects are not yet completely understood. It is well established that under physiological conditions, the SN has unique biochemical features which provide a higher vulnerability to OS (Kidd, 2000) when compared to other brain regions, including the CS (Mythri et al., 2011). The present study was conducted to test the hypothesis that OS can be a potential mechanism involved in the neurodegeneration of SN dopaminergic cells induced by EFA dietary restriction. We tested whether this restriction for one or two generations could induce LP or modify the anti-oxidant activity of SOD or CAT in the SN and CS of rats.

MATERIALS AND METHODS

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 a 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. (1995) and meet all current nutrient standards for rat pregnancy and growth (**Table 1**). The control diet contained 50 g/Kg of soybean oil with adequate amounts of saturated, monounsaturated, α-linolenic (6% of total fatty acids) and linoleic (56% of total fatty acids) acids. The experimental diet contained 50 g/Kg of coconut oil (from Babaçu, *Orbignia martiana*) with reduced levels of linoleic and α-linolenic acids and higher levels of saturated (2-fold) and monounsaturated (2.5-fold) fatty acids (**Table 2**).

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[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 B12 (0.25); vitamin K1 (7.5). Additionally containing (UI%): vitamin A 40.000; vitamin D3 10.000; vitamin E (750).*

[b] *Mineral mixture (Rhoster Ind. Com. LTDA. SP Brazil) containing (m%): CaHP04 (38); K2HP04 (24); CaCO3 (18.1); NaF (0.1); NaCl(7.0); MgO (2.0); MgS04 7H20 (9.0); FeS04 7H20 (0.7); ZnS04 H20 (0.5); MnSO+ H20 (0.5); CuS04 5H20 (0.1); Al2 (S04)3K2S04 24H20 (0.02); Na2SeO3 5H20 (0.001); KCl (0.008).*

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

Fatty acids	Control diet	Experimental 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. Bold values indicate $p < 0.001$.

Rat offspring ($n = 112$) were the object of the present study and only males were used for the experimental assays. Litters were culled to six pups on postnatal day 1 and weaned on postnatal day 21. Dams and pups were distributed into two main groups according to the nutritional condition: control (C) and experimental (E) rats. After weaning, pups were separated and fed *ad libitum* the same diet as their respective mothers. First generation (CF1 and EF1) male rats were weighed and evaluated for biochemical parameters related to LP and anti-oxidant markers at 90–110 days. The remaining males and females were allowed to mate to provide the second-generation groups (CF2 and EF2), which were analyzed at 30–42 days. In each group, animals were sampled randomly 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 6:00 h).

Each experimental day, six animals per group were anesthetized with isofluorane and then decapitated. The regions containing the SN or CS were rapidly dissected in 0.9% (w/v) NaCl solution at 2°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 for the determination of thiobarbituric acid-reactive substances (TBARS) level 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. An aliquot of supernatant was analyzed for total protein content using a bicinchroninic acid protein kit (Sigma-Aldrich, St. Louis, MO).

LIPID PEROXIDATION

LP was measured by estimating malondialdehyde (MDA) using a thiobarbituric acid (TBA) reaction (TBARS method) according to Ohkawa et al. (1979). 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 in a boiling water-bath for 30 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 2500 g for 10 min and the organic phase was read at 532 nm using a plate reader. The results were expressed as nmol per mg of protein using a standard curve generated using different concentrations 1,1,3,3-tetramethoxypropane solution. The control SN and CS samples were incubated in a 30 μM sodium nitroprusside (SNP) solution for 45 min before the assay and used as positive controls for LP.

SUPEROXIDE DISMUTASE (SOD) ASSAY

Assessment of total SOD (t-SOD) enzymatic activity was performed according to Misra and Fridovich, (1972) at 25°C. Triplicates of SN or CS supernatants (100 μL) were previously incubated in a water bath at 37°C and then added to 880 μL of 0.05% sodium carbonate solution pH 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 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 of SN and CS in a 30 μM SNP solution for 45 min before the enzymatic assay.

CATALASE (CAT) ASSAY

CAT activity was measured according to Aebi (1984). 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 catalase activity were obtained by incubation of SN and CS 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.

STATISTICAL ANALYSIS OF OXIDATIVE STRESS PARAMETERS AND BODY WEIGHT

All biochemical experiments were carried out in triplicate and repeated at least twice. Six animals from three litters per group were used each time. A total of 38 and 58 animals were used in the F1 and F2 generations, respectively. Biochemical data of TBARS levels, t-SOD, and catalase enzymatic activity were plotted using GraphPad Prism 5.0 software and the statistical analysis

was performed using ANOVA followed by Tukey as the *post-hoc* test or Student's *t*-test in some cases. The analysis of body weight was carried out using unpaired Student's *t*-test. Differences were considered significant when $p < 0.05$.

FLUORO JADE C (FJC) ASSAY

Considering our recent evidence that a loss of SN dopaminergic cells is induced by EFA dietary restriction for two generations (Passos et al., 2012), FJC, a polyanionic fluorescein derivative, was applied to examine signals of neurodegeneration. It has been shown that this protocol specifically labels damaged neurons and not glial cells in the SN and CS (Bian et al., 2007; Ehara and Ueda, 2009) when these regions are submitted to certain types of insult, especially under conditions that induce OS (Ehara and Ueda, 2009; Li et al., 2009; Yang et al., 2011).

Animals from the F1 and F2 groups ($n = 6$/group) were anesthetized with a sodium pentobarbital solution (100 mg/kg, i.p. Sigma-Aldrich, St. Louis, MO), perfused with a 0.9% NaCl solution, followed by 4% paraformaldehyde in a phosphate buffered saline (PBS), pH 7.4. The brains were post-fixed in the same fixative for two hours, rinsed in a phosphate buffer (PB) and subsequently cryoprotected in 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 in the parasagittal plane. All sections were collected serially in PB and arranged in six series. The Atlas of Paxinos and Watson (1986) was used to delimit cytoarchitectonic regions of interest. Sections of one series per animal were mounted on gelatin-coated slides, air-dried, and subjected to FJC staining according to Ehara and Ueda (2009). Slides were immersed in a 1% NaOH solution (in 80% ethanol) for 5 min, rinsed for 2 min in 70% ethanol, and for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 5 min. After water washing (2 min), the slides were immersed in a FJC solution (0.0001%) in 0.1% acetic acid for 10 min followed by washing in distilled water. The slides were air-dried on a slide warmer at 50°C for 30 min, cleared in xylene, cover slipped with Entellan (Merck). As a positive control for FJC labeling we used brain sections of rats previously treated with the mitochondrial toxin 3-Nitropropionic Acid (3-NP) which induces striatal neurodegeneration. The animals treated with 3-NP were from another study not related to the present work. As a better positive control for FJC labeling in the SN, we used also brain sections of animals which previously received intracerebral injections of pilocarpine in order to induce epilepticus status. The number of FJC-positive neurons was analyzed in the CS and SN in six animals of C and EF2 groups at the stereotaxic coordinate identified as corresponding approximately to lateral 1.9 mm (plate 81) according to Paxinos and Watson (1986).

Double fluorescence staining against FJC and tyrosine hydroxylase in brain sections of two EF2 animals was achieved by the method described by Ehara and Ueda (2009). Tissue sections were incubated first with blocking solution containing 1% BSA, 0.3% triton X-100 for 60 min and then with rabbit polyclonal anti-TH antibody (1:500; Millipore) for 24 h at 4°C. The sections were washed three times in phosphate buffer (PB) 0.1 M, pH 7.4, and incubated for 4 h with Rhodamine-conjugated 546-labeled anti-rabbit IgG (1:600; Jackson). After washing twice in PB, they were mounted onto gelatin coated slides and dried at 50°C for 30 min. The samples were rehydrated for 1 min, incubated in 0.06% potassium permanganate solution for 5 min, and then rinsed for 1 min in distilled water followed by FJC (0.0001% dissolved in 0.1% acetic acid) for 30 min. After rinsed in distilled water, the sections were dried at 50°C for 20 min, cleared in xylene for 1 min and coverslipped with Entellan. Fluoro-Jade C and TH in the CS and SN were analyzed using an epifluorescence microscope (Leica, DM LB).

FATTY ACID DETERMINATION IN THE CORPUS STRIATUM AND MIDBRAIN

The fatty acid profiles of CS and midbrain phospholipids were assessed in F1 groups at 95 days and F2 groups at 35 days of age. The pups ($n = 6$/group) were decapitated and the regions containing the CS or 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 28,000 g at 4°C. The pellets were immediately re-suspended in 50 mM Tris-HCl buffer (pH = 7.4). The total lipids of CS or midbrain homogenates were extracted according to Folch et al. (1957). The phospholipids were then separated by means of a Sep-Pak procedure (Juaneda and Rocquelin, 1985) and transmethylated (Berry et al., 1965). These samples were analyzed using a Shimatzu GC apparatus equipped with a flame ionization detector and HP-inowax 20 M) 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 isoctane extract. A standard fatty acid methyl ester mixture (Supelco™, 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. Data are expressed as the mean ± standard deviation (SD). Differences between the groups were analyzed by Student's *t*-test and considered significant at $P < 0.05$.

RESULTS

Data on body weights of F1 and F2 groups are presented in **Table 3**. Adult animals of the EF1 group and young animals of the EF2 group showed significantly lower body weights when compared to the control ($p < 0.05$).

Table 3 | Body weights of F1 and F2 animals.

Groups	Body Weight (g)	
CF1	402.54 ± 40.04	($n = 38$)
EF1	376.97 ± 36.92**	($n = 43$)
CF2	79.65 ± 14.87	($n = 33$)
EF2	71.91 ± 10.09*	($n = 43$)

Values are expressed as Mean ± SD.

*$P < 0.05$; **$P < 0.01$ Unpaired Student's t-test.*

CORPUS STRIATUM AND MIDBRAIN FATTY ACID PROFILE

Table 4 shows the midbrain fatty acid profile of F1 generation adult animals and **Table 5** combines data of midbrain and CS fatty acids of the F2 generation young animals raised under either control or experimental diets. As can be observed, the midbrain phospholipids from the EF1 and EF2 groups exhibit, respectively, 28 and 50% lower DHA levels (22:6n–3) as compared to their control groups. DHA levels were also lower in the EF2-CS phospholipids (~50%) when compared to control. The reduced levels of DHA in both EF1 and EF2 groups was accompanied by a significant increase in the docosapentanoic fatty acid (DPA; 22:5n6) contents (2-tail *t*-test, $P < 0.001$). On the other hand, the values for AA (20:4n–6) did not differ between both groups of F1 or F2 generations. With respect to saturated and monounsaturated fatty acids, the presence of coconut oil in the maternal diet significantly increased the levels of palmitic (16:0), stearic (18:0), palmitoleic (16:1), and oleic (18:1n9) acids (2-tail *t*-test, $P < 0.01$) in the EF2 midbrain phospholipids.

Table 4 | Fatty acid composition (% of total) in midbrain phospholipids of F1 generation groups raised on Control or Experimental diets.

Fatty acid	Midbrain	
	Control diet	Experimental diet
C16	16.41 ± 1.9	15.85 ± 0.81
C16:1	0.96 ± 0.34	1.10 ± 0.28
C18	22.47 ± 1.63	23.99 ± 1.67
C18:1n9	24.55 ± 0.96	24.64 ± 2.00
C20	0.74 ± 0.13	0.91 ± 0.10
C20:1	2.04 ± 0.21	2.83 ± 0.12
C20:4n6	8.76 ± 0.32	8.73 ± 0.30
C20:3n6	0.45 ± 0.30	0.35 ± 0.24
C22	0.97 ± 0.53	0.80 ± 0.11
C23	3.11 ± 0.51	3.10 ± 0.52
C22:5n6	1.03 ± 0.74	3.16 ± 0.75***
C22:6n3	14.41 ± 1.81	11.25 ± 0.69**
C24:1n	2.24 ± 0.52	1.07 ± 0.18

Values are expressed as means ± SD.
***p < 0.01; ***p < 0.001.*

LIPID PEROXIDATION AND T-SOD ENZYME ACTIVITY IN ADULT ANIMALS OF F1 GENERATION

Biochemical results of the F1 groups are summarized in **Figure 1**. As expected, LP (measured as TBARS levels) was found to be significantly increased in SN (0.770 ± 0.136 nmol MDA/mg protein) and CS (0.834 ± 0.140 nmol MDA/mg protein) homogenates of CF1 group previously treated with 30 μM SNP, compared to the control condition (0.425 ± 0.105 and 0.532 ± 0.015 nmol MDA/mg protein for SN and CS, respectively; $P < 0.001$). However, TBARS levels in both regions were not modified in rats fed on the experimental diet (0.494 ± 0.089 and 0.570 ± 0.038 nmol MDA/mg protein for SN and CS, respectively) when compared to the control animals (**Figure 1A**). Consistent with these results, a significant increase in the t-SOD enzyme activity was observed in the EF1 group ($P < 0.01$) either in the SN (0.735 ± 0.020 U/mg protein) or CS (0.640 ± 0.192 U/mg protein) compared to the control condition not submitted to pre-treatment with SNP (0.606 ± 0.028 and 0.355 ± 0.034 U/mg protein for SN and CS, respectively). As can be observed, the SNP treatment used as a positive control, significantly increased SOD activity in the SN (1.241 ± 0.206 U/mg protein) and CS (1.832 ± 0.046 U/mg protein).

LIPID PEROXIDATION, T-SOD, AND CAT ENZYME ACTIVITIES IN YOUNG ANIMALS OF F2 GENERATION

In young animals of the F2 generation, distinct effects were induced by the experimental diet in the two regions analyzed. Evidence of LP, assessed by a significant increase in TBARS levels, was detected in the SN of EF2 group (0.564 ±

Table 5 | Fatty acid composition (% of total) in Corpus Striatum and Midbrain membrane phospholipids of F2 generation groups raised on Control or Experimental diets.

Fatty acid	Corpus Striatum		Midbrain	
	Control diet	Experimental diet	Control diet	Experimental diet
C16	17.99 ± 1.04	21.74 ± 0.74	16.51 ± 1.90	24.09 ± 0.27*
C16:1	0.73 ± 0.10	0.73 ± 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	25.20 ± 0.51	24.88 ± 0.67	29.09 ± 0.37*
C18:1n9	17.07 ± 0.34	15.18 ± 0.69	16.38 ± 0.25	19.21 ± 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)	14.06 ± 0.27	13.12 ± 0.34	13.74 ± 0.83	14.93 ± 0.48
C23	3.66 ± 0.13	3.46 ± 0.18	3.96 ± 0.20	3.52 ± 0.19
C22:5n6	1.54 ± 0.06	9.60 ± 0.26**	1.49 ± 0.11	9.39 ± 0.31**
C22:6n3 (DHA)	19.23 ± 0.88	9.48 ± 0.84**	19.70 ± 0.69	8.70 ± 0.73**

Values are expressed as means ± SD.
**p < 0.01; **p < 0.001; nd, not detected.*

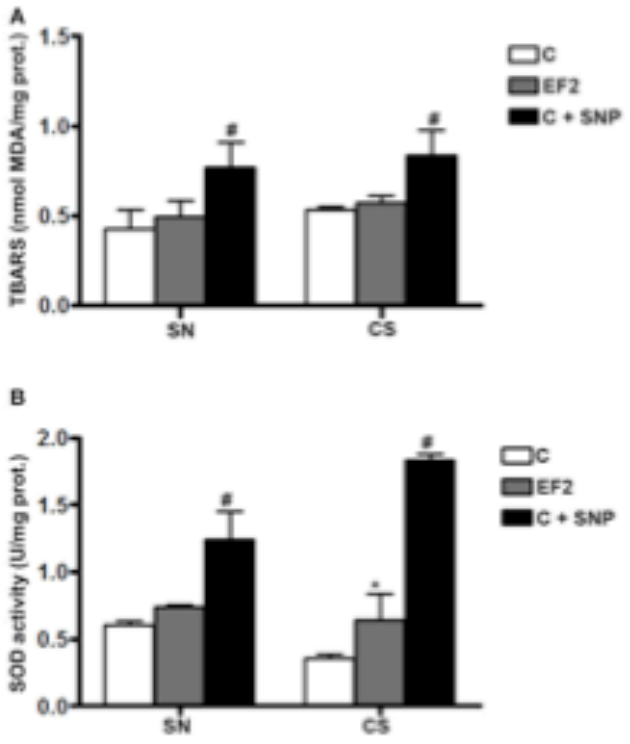

FIGURE 1 | Thiobarbituric acid-reactant substances (TBARS) levels (A) and total superoxide dismutase (t-SOD) activities (B) in the pool of Substantia Nigra and Corpus Striatum from first generation adult rats fed essential fatty acid restricted diet and controls ($n = 12$ per group). $^{*}P < 0.05$ compared to control group. Treatment of control homogenates with sodium nitroprusside (SNP) was used as positive control in all the experiments. #$P < 0.001$ compared to control or EF1 groups.

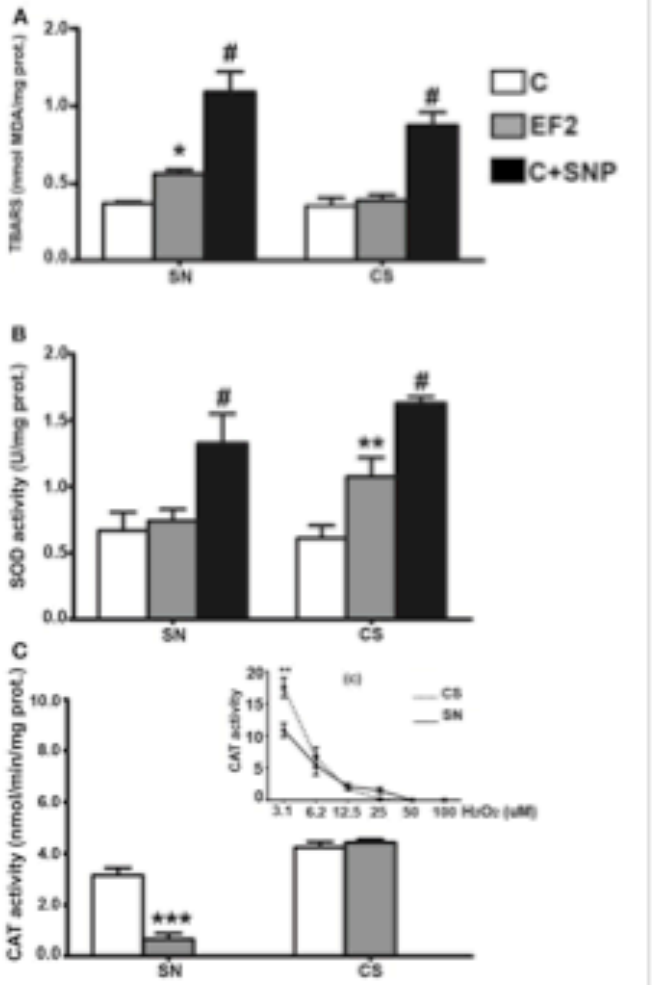

FIGURE 2 | Thiobarbituric acid-reactant substances (TBARS) levels (A), total superoxide dismutase (t-SOD) activities (B), and catalase (CAT) activities (C) in the pool of Substantia Nigra and Corpus Striatum from young rats fed an essential fatty acid restricted diet over two generations and respective controls ($n = 12$ 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 for TBARS and t-SOD in all the experiments. The insert (c) in the panel **(C)** shows the H_2O_2 concentration-dependent manner of the CAT activity in the SN and CS control homogenates obtained as positive controls. #$P < 0.0001$ compared to control or EF2 groups.

0.02 nmol MDA/mg protein) in comparison with the control group (0.372 ± 0.01 nmol MDA/mg protein, $P < 0.05$). The magnitude of LP induced by the experimental condition in the SN is about 50% less than that obtained by using 30 μM SNP (1.330 ± 0.220 nmol MDA/mg protein). No difference between the EF2 (0.354 ± 0.005 nmol MDA/mg protein) and the C (0.391 ± 0.083 nmol MDA/mg protein) groups was found in the CS (**Figure 2A**). A significant increase in t-SOD enzyme activity was found in the CS of the EF2 group (1.074 ± 0.145 U/mg protein) compared to the control group in the absence of pretreatment with SNP (0.610 ± 0.096 U/mg protein, $P < 0.01$). Nevertheless, the increase in SOD activity in the EF2 group CS was smaller than that induced by 30 μM SNP in the C group (1.633 ± 0.046 U/mg protein). No difference between the groups was detected for t-SOD activity in the SN (0.741 ± 0.087 and 0.667 ± 0.138 U/mg protein for the EF2 and C groups, respectively) as shown in the **Figure 2B**. On the other hand, the CAT activity was significantly reduced in the SN of the EF2 group (0.652 ± 0.238 nmol/min/mg protein) compared to the control group (3.159 ± 0.279 nmol/min/mg protein in the control; $P < 0.001$). No difference between the groups was detected in CAT activity in the CS (4.339 ± 0.217 nmol/min/mg protein and 4.420 ± 0.125 nmol/min/mg protein for the EF2 and C groups, respectively) as shown in **Figure 2C**. The insert in the **Figure 2C** shows the H_2O_2 concentration-dependent manner of the CAT activity in the SN and CS control homogenates obtained as positive controls. As can be observed, at lower concentrations of H_2O_2, the CAT activity is significantly greater in the CS as compared to SN ($p < 0.05$) but this difference disappears at higher concentrations.

FLUORO JADE C AND TYROSINE HYDROXILASE LABELING

Fluoro-Jade C-positive cell bodies were not detected in the SN or CS in the groups (6 animals/group) of F1 generation (**Figure 3A**). In the SN of the EF2 group, several FJC-positive cells were seen either in the pars compacta or in the pars reticulata while no

labeling was detected in cell bodies of the CS in all animals ($n = 6$) analyzed (**Figure 3B**). In the EF2 group ($n = 6$), the number of FJC-positive cells distributed in the *pars compacta* and *pars reticulata* at the middle level of SN changed from 59 to 70 cells and the average number was estimated as 63.8 ± 6.4 cells.

Double fluorescence staining for FJC and TH of a representative EF2 animal is shown in the **Figure 4**. As can be seen, signals of degeneration were detected in SN dopaminergic and non-dopaminergic neurons either in the *pars reticulata* or in the *pars compacta*. Nevertheless, no staining for FJC was found in cell

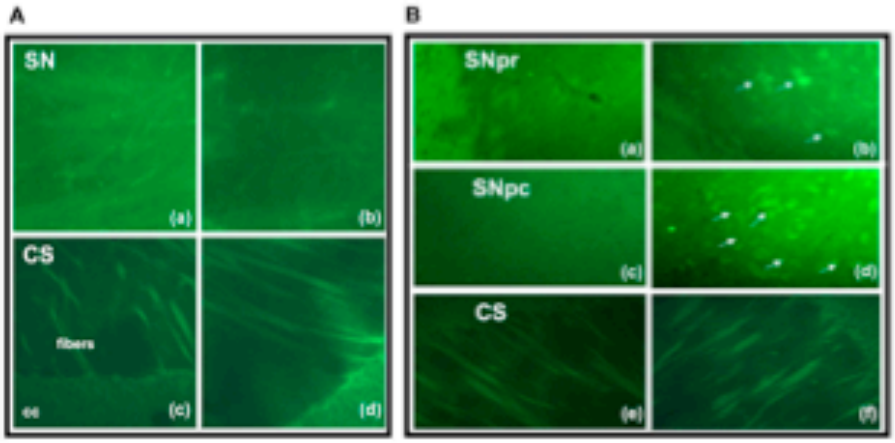

FIGURE 3 | Fluoro-Jade C staining in brain parasagittal sections of F1- (panel A) or F2- (panel B) generation groups at the level of Substantia nigra (SN) or Corpus Striatum (CS). No labeling was detected in cell bodies or processes of SN **(Aa, Ab)** and CS **(Ac, Ad)** in adult animals of F1 generation. However, FJC positive cell bodies and processes were detected in the SN pars reticulata **(Bb)** and pars compacta **(Bd)** of EF2 group while no labeling in these regions was seen in the controls **(Ba, Bc)**. No FJC labeling was detected in cell bodies of the CS in the EF2 **(Bf)** or control **(Be)** groups. A slight and non-specific labeling was seen in regions rich in myelin such as cerebral peduncle (cp), corpus callosum (cc), or myelinated fibers crossing the CS.

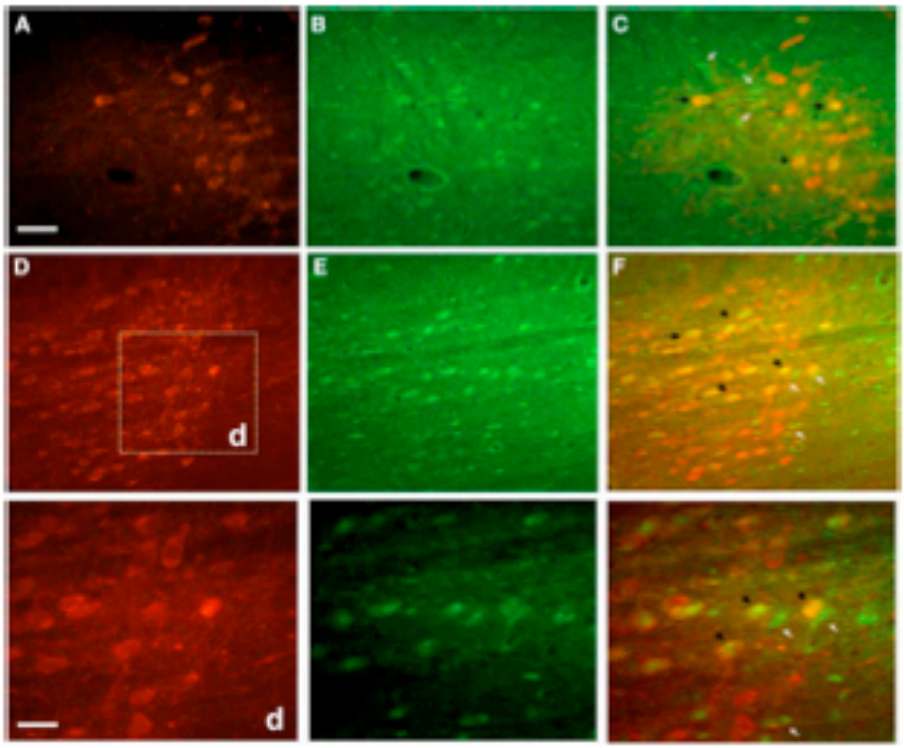

FIGURE 4 | Photographs of epifluorescence microscopy showing SN sections from a representative EF2 animal subjected to TH immunostaining followed by Fluoro-Jade C staining. Examples of single (FJC; yellow arrows) or double (TH + FJC; black arrows) labeled cells can be seen either in the SN *pars reticulata* (**A**,**B**, and **C**) or in the *pars compacta* (**D**,**E**, and **F**). High magnification of the region (d) is shown in the bottom panel. Scale bar of **A** = **B**,**C**,**D**,**E**, and **F** represents 60 μm while the scale bar of bottom panel represents 20 μm.

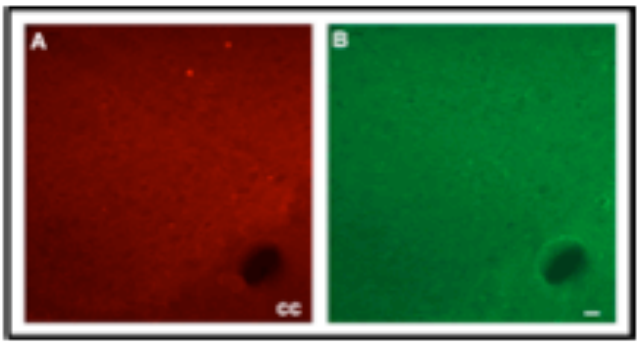

FIGURE 5 | Photographs of epifluorescence microscopy showing CS sections from a representative EF2 animal subjected to TH immunostaining (A) followed by Fluoro-Jade C staining (B). Note the absence of FJC-positive cells surrounded by TH-positive neuronal terminals. cc = corpus calosum. Scale bar = 40 μm.

bodies surrounded by TH-positive neuronal terminals in the CS, confirming data obtained using single labeling for FJC (**Figure 5**).

DISCUSSION

The current study investigated whether a dietary restriction of both linoleic and α-linolenic fatty acids for one or two generations could affect the redox balance in the SN and CS. We hypothesized that OS could be a potential mechanism involved in the loss of dopaminergic cells previously demonstrated (Passos et al., 2012). Our data showed signals of degeneration in SN dopaminergic and non-dopaminergic neurons and indicated a differential vulnerability of SN and CS to oxidative insult induced by two generations of EFA dietary restriction.

REPERCUSSION OF DIETARY TREATMENT ON BODY WEIGHT

The significant lower body weight gain of adult EF1 and young EF2 animals is in agreement with previous studies using coconut oil as the only source of dietary lipids (Deuel et al., 1954; Soares et al., 1995; Borba et al., 2010). Regarding this effect, this type of dietary treatment has been associated with dysfunction of growth hormone regulation (Soares et al., 1995). Moreover, it has been reported that coconut oil can reduce body weight due to high saturated medium chain fatty acids (8:0–14:0) turnover rates, which are predominant in its lipid profile. Such effect seems to be independent of essential fatty acid deficiency (Hargrave et al., 2005).

MIDBRAIN AND CORPUS STRIATUM FATTY ACID PROFILE

It has been demonstrated that a diet containing coconut oil as the only source of lipids depletes DHA in the brain more than a fat free diet, even for a short-term treatment, especially due to the diet's high content of saturated fatty acids (Ling et al., 2010). In the present study, the experimental diet based on coconut oil significantly reduced DHA levels about 28 and 50% in the midbrain phospholipids of the EF1 and EF2 groups, respectively, as compared to their controls. The DHA depletion was accompanied by a significant increase in DPA levels, which reinforces the condition of DHA deficiency. On the other hand, despite containing 8% linoleic acid (about 30% of recommended minimal dietary requirement for rodents (Bourre et al., 1990), the experimental diet did not modify the AA levels in either region of EF2 group. These results agree with other studies, indicating that AA is more tightly controlled than DHA in the central nervous system and that its brain concentrations are less vulnerable to limitations in the supply of precursor than other organs (Bourre et al., 1990; Brenna and Diau, 2007; Igarashi et al., 2009; Ling et al., 2010). In fact, recent evidence has indicated that even when using a diet containing 2.3% linoleic acid for 15 weeks, starting at weaning, the brain AA concentration is reduced by only 28%, while a 74% reduction has been observed in the liver of the same rats (Igarashi et al., 2009). Thus, in addition to DHA deficiency, our dietary treatment was able to increase AA/DHA ratio in the fatty acid profile of SN and CS phospholipids.

REPERCUSSION OF DIETARY TREATMENT ON LIPID PEROXIDATION AND ENZYMATIC ANTI-OXIDANT ACTIVITY

It has been established that an imbalance in the AA/DHA ratio and especially DHA deficiency can decrease anti-inflammatory and anti-oxidant responses and induce cellular damage in different classes of neurons (Yavin, 2006; Schmitz and Ecker, 2008). An inverse relation between the number of some brain neurons and increasing ratios of n–6/n–3 EFAs in the maternal diet has been also recently reported (Tian et al., 2011). In the present study, an increase in the t-SOD activity observed in the SN and CS of the EF1 group was able to protect these regions from membrane LP measured as TBARS levels. The absence of FJC labeling in neuronal cell bodies of both brain regions reinforces these results, considering the efficacy of this reagent in detecting signals of neurodegeneration induced by conditions of OS, such as ischemia (Yang et al., 2011), glutamate excitotoxicity (Ehara and Ueda, 2009) or dopaminergic lesions induced by 6-OHDA (Ehara and Ueda, 2009) or MPTP (Bian et al., 2007; Li et al., 2009).

EFA dietary restriction over two generations, which induced a more expressive DHA deficiency in midbrain phospholipids (~50%) and AA/DHA ratio (~2), was able to provoke LP and impaired the anti-oxidant responses at least in SOD and CAT enzymes in the SN of the EF2-group as compared to the control. Such results are consistent with recent evidence of the protective action of DHA dietary supplementation on SN cell populations under experimental conditions that induce OS, such as MPTP (Ozsoy et al., 2011). The lack of efficient t-SOD reactivity and the expressive reduction in the CAT activity observed in the EF2 group shows the vulnerability of SN to conditions that reduce DHA availability during the critical period of brain development. Studies on rats or human SN have indicated a progressive decrease in the activity of several anti-oxidant enzymes including SOD and CAT during physiological brain aging (Kolosova et al., 2003; Venkateshappa et al., 2012). The present findings in the EF2 young animals corroborate our initial hypothesis indicating that a decreased anti-oxidant function can be a potential mechanism by which long-term EFA dietary restriction induces loss of SN dopaminergic neurons (Passos et al., 2012). Thus, increased levels of OS in the young brain might act synergistically with other deleterious effects induced by DHA deficiency, accelerating the degenerative profile of SN. The FJC staining in the SN of EF2 animals reinforces these data, demonstrating the presence of

neuronal damage in several dopaminergic neurons either in the *pars compacta* or in the *pars reticulata*. Moreover, we also detected signals of degeneration in non-dopaminergic cells at the same regions of SN, suggesting that the oxidative insult induced by EFA dietary restriction affects neuronal populations with distinct neurochemical profile.

In contrast to the effects detected in the SN and despite a similar DHA deficiency, we did not observe LP or anti-oxidant dysfunction in the CS of the EF2 young rat brains, when compared to their respective controls. In support of this biochemical data, we did not find FJC-positive cell bodies in parasagittal or transversal sections of this nucleus. These findings reinforce some early and recent evidence in human and experimental animals that this region is more resistant than SN under physiological (Kolosova et al., 2003; Venkateshappa et al., 2012) or pathological conditions where SN dopaminergic neurons are affected (Floor and Wetzel, 1998; Mythri et al., 2011). The significant increase in the t-SOD activity in the CS of the EF2 animals indicates that this region has differential compensatory means which can be triggered from the insult induced by DHA deficiency. It is noteworthy that under normal conditions, dietary DHA supplementation, even for a short period (30 days), is able to increase the t-SOD activity in the CS of adult rats, which has been suggested as a potential regulatory action of this LC-PUFA on this enzyme (Sarsilmaz et al., 2003). If this is the case, our findings suggest that such action could be activated even under conditions of 50% DHA depletion in the CS phospholipids. A differential reactivity of CS under OS conditions was also recently reported: in animals injured with 6-OHDA, the dopamine turnover is significantly increased in this nucleus by fish oil supplementation (Delattre et al., 2010).

The increased t-SOD activity in the CS was not accompanied by a similar CAT reaction, which did not change its activity as compared to the control condition. These enzymes play complementary activities in the anti-oxidative defense system, considering that the H_2O_2 generated by SOD activity is the substrate for CAT. Thus, the absence of LP in the CS suggests that other anti-oxidant mechanisms involved in the degradation of H_2O_2 could be implicated in the relative resistance of this nucleus. An expressive increase in the total glutathione levels and in the glutathione peroxidase activity associated with glial cell proliferation has been found in the CS and frontal cortex of human postmortem PD brains (Mythri et al., 2011). Although future studies need to be carried out in order to address this issue in our experimental model, preliminary results of our group indicate that the glial cell reactivity might be also implicated in the lower vulnerability of CS to oxidative insult described herein.

Despite the resistance of CS to OS under the present experimental conditions, we cannot discard potential effects of EFA dietary restriction on other parameters involving neuroprotection in the nigrostriatal system. The anti-oxidant parameters here investigated under conditions of DHA deficiency probably are not working alone. A recent study demonstrated that a relatively short-term feeding of an α-linolenic acid-restricted diet was able to lower the DHA content and the brain derived neurotrophic factor (BDNF) levels in the mouse striatum, while two other brain regions were not affected (Miyazawa et al., 2010). Conversely, when DHA is supplemented in the diet, CS strongly reacts to OS induced by MPTP, increasing the synthesis of BDNF more than in control conditions (Bousquet et al., 2009). The disrupted relation between OS and neurotrophin availability could be involved in behavioral or neurochemical effects observed in animals or human beings submitted to EFA dietary restriction (Fedorova and Salem, 2006; Kuperstein et al., 2008).

CONCLUSION

The present data shows the importance of adequate dietary levels of EFA to maintain an effective redox balance in the SN. Our results demonstrate that LP associated with an impaired anti-oxidant response increases the vulnerability of SN dopaminergic and non-dopaminergic neurons to degeneration induced by long-term EFA dietary restriction. These results reinforce the hypothesis that this dietary treatment increases the risk of certain neurological disorders. The data also demonstrate that biological mechanisms of resilience can be activated in the CS even under a similar condition of DHA deficiency.

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Substantia nigra neurodegeneration induced by DHA deficiency involves increased nitric oxide levels in the corpus striatum of adult rats

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ABSTRACT

The vulnerability of nigro-striatal dopaminergic neurons to lesions is under discussion in the literature due to the relevance of these cells under physiological or pathological conditions. Our previous study on young rat brains demonstrated that essential fatty acid dietary restriction over two generations induced neurodegeneration in substantia nigra (SN) dopaminergic and non-dopaminergic cells, increased levels of lipoperoxidation (LP) and reduced enzymatic anti-oxidant resource in the SN but not in the corpus striatum (CS). In the present study, using the same experimental model, we investigated whether increased nitric oxide levels could contribute to SN oxidative stress (OS) in young animals and extended our analysis to adult animals. We also hypothesized that increasing omega-3 deficiency in adult animals could reduce the number of BDNF expressing neurons in the SN and the redox balance in the CS. Second generation (F2) rats were raised from conception on control or experimental diets containing adequate or reduced levels of linoleic and α-linolenic fatty acids, respectively. Lipid peroxidation (LP) was measured using the thiobarbituric acid reaction method (TBARS), nitric oxide production was evaluated via nitrite concentration and the total superoxide dismutase (t-SOD) and catalase (CAT) enzymatic activities were assessed in SN and CS homogenates. Long-term treatment with the experimental diet modified the fatty acid profile of SN and CS phospholipids and significantly decreased the number of TH-positive cells in the rostro-dorso-medial (~50%) and caudo-ventro-lateral (~40%) regions of SN and the total number of BDNF immunoreactive neurons (~20%). Increased NO levels were observed in the CS of both young (~30%) and adult (~1.8 fold) experimental animals while no inter-group difference was found for this parameter in the SN. A significant increase in LP ($p<0.01$) and decrease in the CAT and t-SOD activity ($p<0.001$) were detected in the CS of experimental group. In this same group, LP and reduced CAT activity was found in the SN but t-SOD activity did not change compared to control. The results show that EFA dietary restriction over two generations until adult stage reduce resilience in the CS increasing LP associated to high a concentration of NO and reduced anti-oxidant activity. Taken together, these parameters worsen dopamine cell loss in the SN affecting also the distribution of BDNF expression.

Keywords: BDNF, lipoperoxidation, DHA, malnutrition, oxidative stress, ganglia basal, tyrosine hydroxilase, superoxide dismutase, catalase, nitric oxide.

INTRODUCTION

The vulnerability of nigro-striatal dopaminergic neurons to lesions has been a matter of discussion and investigation in early and recent studies, especially due the relevance of these cells in the etiology of Parkinson's disease (HASSLER, 1938; GONZALEZ-HERNANDEZ et al., 2010). It is well established that under physiological conditions, the substantia nigra (SN) has unique biochemical features which provide a higher vulnerability to oxidative stress (OS) when compared to other brain regions (KIDD, 2000). 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 (GONZALEZ-HERNANDEZ et al., 2010).

The SN exhibits a high concentration of microglia (LAWSON et al., 1990), the over-activation of which can result in the release of cytokines and free radicals such as superoxide radicals and nitric oxide (NO) (MINGHETTI et al., 1999). These bioactive molecules released from microglia have been thought to contribute to SN dopaminergic cell death induced by mitochondrial dysfunction (DEPINO et al., 2003; ZHANG *et al.,* 2006) or by lipopolysaccharide induced inflammation (ARIMOTO and BING, 2003). An intrinsic neuronal population containing nitric oxide synthase (NOS) and nitrergic afferent neurons from the pedunculopontine tegmental nucleus (PPTg) are also present in the SN (Gonzalez-Hernandez et al., 1997). The potential involvement of neuronal NOS (nNOS) activity in nigral cell degeneration has been discussed because of the importance of NO as a physiological modulator of cortico-striatal glutamatergic activation and since its synthesis in the corpus striatum (CS) can be modulated by dopamine receptor subtypes D1 and D2 (GONZALEZ-HERNANDEZ et al., 1997; CALABRESE *et al*., 2007; WEST and TSENG, 2011).

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 and nucleus accumbens (DELION et al., 1994; ZIMMER et al., 2000; CHALON, 2006) and dopaminergic cell loss in the substantia nigra (SN) pars compacta (Ahmad et al., 2008; Passos et al., 2012) have been described as a consequence of this nutritional deficiency. Conversely, in experimental models of Parkinson disease, dietary DHA or fish oil supplementation have been partially able to restore dopaminergic neurotransmission after 6-hydroxidopamine (6- OHDA) – or 1-metil-4-fenil-1,2,3,6-tetrahydropyridine (MPTP)- induced striatal lesions (BOUSQUET et al.,

2008; CANSEV et al., 2008). Recent studies have also linked DHA and the expression of neurotrophins involved in the development and survival of midbrain neurons. For example, in one study, dietary DHA supplementation was able to increase neurotrophin levels such as glial cell-derived neurotrophic factor (GDNF) and neurturin (NTN) in the SN, reducing dopaminergic cell death induced by MPTP (TANRIOVER et al., 2010), as well as increased brain-derived neurotrophic factor (BDNF) mRNA expression in mouse corpus striatum (BOUSQUET et al., 2009). The influence of DHA on BDNF levels and/or activity of its receptor TrkB have been also reported in the hippocampus, cerebral cortex (VINES et al., 2012) and spinal cord (YING et al., 2012)

Among other factors with SN activity, only BDNF is both a potent dopaminergic and GABAergic neurotrophin (HYMAN et al; 1991, STAHL et al., 2011) and normally expressed in high levels in the adult nigrostriatal system (GAL et al., 1992; ABE et al. 2010) exerting also neuroprotection in the aging brain (SINGH et al., 2006; BORGER et atl., 2011). Although mRNA for BDNF is present in the corpus striatum, experimental studies using colchicine treatment demonstrated that BDNF is produced in the SN and is anterogradely transported to the CS (ALTAR and DISTEFANO, 1998). 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 (HYMAN et al; 1991; SHULTS et al., 1995; STAHL et al., 2011). 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 (TSUKAHARA et al., 1995). Evidence in humans has demonstrated that BDNF mRNA expression in the SN (HOWELLS et al., 2000) and BDNF protein levels in the caudate, putamen and SN (MOGI et al., 1999; PARAIN et al., 1999) are reduced in patients with Parkinson`s disease. Furthermore, chronic deprivation of TrkB signaling leads to selective late onset nigrostriatal dopaminergic degeneration (BAYDYUK et al., 2011)

Previous studies from our laboratory, adopting a two-generation model of essential fatty acid (EFA) dietary restriction, demonstrated that this type of malnutrition was able to significantly reduce 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 (SNcv) region of SN in young animals (PASSOS et al., 2012). Analyzing potential mechanisms involved in these deleterious effects, we demonstrated that EFA dietary restriction induced signs of neurodegeneration in SN dopaminergic and non-dopaminergic neurons, increased levels of lipoperoxidation (LP) and reduced enzymatic anti-oxidant

resource in the SN but not in the CS (CARDOSO et al., 2012). 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 is crucial for building neural resilience during adulthood, we also hypothesized that increasing DHA deficiency in adult animals could affect dopaminergic neurons in the SNcv and the redox balance in the CS. Furthermore, taking into account the importance of paracrine action of BDNF on SN dopaminergic and non-dopaminergic cells, we also analyzed in this experimental model how the number of SN BDNF positive neurons could be affected by EFA dietary restriction.

Materials and methods

Animals, diet 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 a control or experimental diets, each containing approximately 400 Kcal/100g and differing only in the lipid source. The diets were prepared according to Soares et al. (1985) and met all current nutrient standards for rat pregnancy and growth as shown in Table 1. The control diet contained 50 g/Kg of soybean oil with adequate amounts of saturated, monounsaturated, a-linolenic (6% of total fatty acids) and linoleic (56% of total fatty acids) acids. The experimental diet contained 50 g/Kg of coconut oil (from Babaçu, *Orbignya martiana*) with reduced levels of linoleic and a-linolenic acids and higher levels of saturated (2 fold) and monounsaturated (2.5 fold) fatty acids (Table 2).

Please, insert Tables 1 and 2 around here

Rat offspring (n=112) were the object of the present study, but only the males were used for the experimental assays. Litters were culled to 6 pups on postnatal day 1 and weaned on postnatal day 21. Dams and pups were distributed into two main groups according to the nutritional condition: control (C) and experimental (E) rats. After weaning, pups were separated and fed *ad libitum* with the same diet as their respective mothers. First generation males and females were

allowed to mate at 30-42 days, to provide the second generation young animals (CF2Y and EF2Y groups) and adult animals at 90-110 days (CF2A and EF2A groups). In each group, animals were sampled randomly 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, six animals per group were anesthetized with isofluorane and then decapitated. The regions containing the SN or CS were rapidly dissected in a 0.9% (w/v) NaCl solution at 2°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 1,000 g at 4°C for an analysis of lipid peroxidation via determination of thiobarbituric acid-reactive substances (TBARS) 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 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). All biochemical experiments above described were carried out in triplicate and repeated at least twice

Lipid Peroxidation

Lipid peroxidation was measured by estimating malondialdehyde (MDA) using a thiobarbituric acid (TBA) reaction (TBARS method) according to Ohkawa et al. (1979). 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 2,500 g for 10 min and the organic phase was read at 532 nm using a plate reader .The results were expressed as nmol per mg of protein using a standard curve generated using different concentrations of 1,1,3,3-tetramethoxypropane solution. The control SN and CS samples were incubated in a 30 μM sodium nitroprusside (SNP) solution for 45 min before the assay and used as positive controls for lipid peroxidation.

Total superoxide Dismutase (t-SOD) activity

Assessment of total SOD (t-SOD) enzymatic activity was performed according to Misra and Fridovich, (1972) at 25°C. Triplicates of SN or CS supernatants (100 μL) had been previously incubated in a water bath at 37°C and then added to a 880 μL solution of 0.05% sodium carbonate with a pH of 10.2 in 0.1mM 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 also expressed as units per milligram of protein (U/ mg protein). Positive controls were obtained by incubating control homogenate samples of SN and CS in a 30 μM SNP solution for 45 min before the enzymatic assay.

Catalase (CAT) activity

CAT activity was measured according to Aebi (1984). 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 $x10^7$, 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 catalase activity were obtained by incubation of SN and CS 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.

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, (1982). 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, Sigma) were placed in 96 well plates and reacted for 10 min at room temperature (~20ºC). The absorbance of diazonium compound was measured at a wavelength of 540 nm. The results were expressed as μmol nitrite per mg of protein with reference to a standard curve built with sodium nitrite concentrations.

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 a 0.1 M phosphate buffer (PB), pH 7.4 (200 ml). Perfusion was always performed between 12:00 and 18:00, with a continuous infusion pump (Harvard equipment) at a perfusion rate of 7.64 ml/min. 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 solutions of 10%, 20% and 30% sucrose in PB. Brain blocks were serially cut on a criostate (Leica) into 50 µm-thick sections across the parasagittal plane, throughout the latero-medial extent of each hemi-brain. All sections were collected serially in PB and arranged in six series. The Paxinos and Watson stereotaxic atlas (1986) was used to delimit the cytoarchitectonic regions. The series of sections used for BDNF or TH immunohistochemistry were treated with a 0,1M borate buffer, pH 9.0, at 60° C for 1h and 0.01 M citrate buffer, pH 6.0, at 60° C for 1 h, respectively. Thereafter, free-floating sections were rinsed in PB and incubated with a rabbit anti-BDNF polyclonal antibody (Santa Cruz, USA; 1:200) and 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 (NGS) for 24 h at 4º C. Sections were then incubated for 1h 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 slides. These procedures were carried out simultaneously in brain sections from both the 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 CF2 and EF2 adult animals was achieved in order to identify the distribution of double and non-double labeled cells in the SNrm and SNcv regions. Two protocols were adopted: in the first one, 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 24h. In the second, double fluorescence was obtained using mouse-monoclonal anti-TH labeled with DyLight-conjugated 488-labeled anti-mouse IgG (1:1000, Rockland) and rabbit polyclonal anti-BDNF with DyLight-conjugated 546-labeled anti-rabbit IgG (1:1000, Rockland) for 24h. After incubation with the fluorophore-coupled antibodies above described, the sections were washed three times in a phosphate buffer (PB) 0.1M, pH 7.4, mounted onto gelatin coated slides, dried at 50°C for 30 min, cleared in xylene for 1 min and mounted with Entellan (Merk). The sections were then analyzed using an epifluorescence microscope (Leica, DM LB).

Quantification of TH and BDNF positive cells in the substantia nigra

Estimates of the number of BDNF and TH-immunoreactive cells in the SNrm and SNcv of adult animals were obtained from six brains per group. The quantitative analysis was performed in three parassagital sections at the middle level of SN, starting from the stereotaxic coordinate corresponding approximately to latero-medial (plate 81 from Paxinos and Watson, 1986). These sections were from one of 6 series obtained from the left side of the brain. A Leica DMLS microscope coupled to SAMSUNG high level color camera (model SHC-410NAD) was used to obtain digital images from immunoreacted brain sections (40 X objective plus 2 X magnification of the camera). Adjacent sampling windows throughout the extension of SNrm and SNcv were analyzed using Image J 1.46 (NIH, USA) software.

Soma size of dopaminergic cells

Cell body areas of TH-immunoreactive neurons in the SNrm or SNcv of adult animals were measured using Image J 1.46 (NIH, USA) software. To delimit the outlines of cell somata, a systematic random sampling of cells was made using high magnification images 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 parassagital 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.

Fatty acid determination in the Corpus Striatum and Midbrain

The fatty acid profiles of 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 28,000 g at 4 °C. The pellets were immediately re-suspended in 50 mM Tris-HCl buffer (pH = 7.4). The total lipids of midbrain homogenates were extracted according to Folch et al., (1956). The phospholipids were then separated by means of a Sep Pak procedure (Juaneda and Rocquelin, 1985) and transmethylated (Berry et al., 1965). These samples were analyzed using a Shimatzu GC apparatus equipped with a flame ionization detector and HP-inowax 20 M capillary column (30 m x 0.32 mm x 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 isoctane 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. Data are expressed as the mean ± standard deviation (SD). Differences between the groups were analyzed by Student's t test and considered significant at $P < 0.05$.

Statistical analysis

Six animals from three litters per group were used each time. A total of 25 young and 87 adult animals were used. Biochemical data of TBARS levels, t-SOD and catalase 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 nonparametric 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 teste, $a<0.05$ was further utilized to determine post hoc comparison among groups and regions.

RESULTS

Body and Brain weights

Body weights of young and adult animals were significantly smaller in the EF2 groups as compared to the respective controls. Brain weights of young but not of adult animals were significantly smaller in the EF2 group, compared to control. Nevertheless, the brain weight/body weight ratio did not differ between the groups in each age (Table 3).

Please, insert Table 3 around here

Corpus striatum and midbrain fatty acid profile

Table 4 combines data of SN and CS fatty acids of the F2 generation adult animals raised under either control or experimental diets. As can be observed, the SN and CS phospholipids from the EF2 groups exhibit ~65% lower DHA levels (22:6n-3) as compared to their control groups. The reduced levels of DHA in the EF2A groups were accompanied by a significant increase in the docosapentaenoic fatty acid (DPA; 22:5n6) contents (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. With respect to saturated and monounsaturated fatty acids, the presence of coconut oil in the maternal diet significantly increased the levels of palmitic (16:0), stearic (18:0), palmitoleic (16:1) and oleic (18:1n9) acids (2-tail t-test, $P < 0.01$) in both SN and CS phospholipids.

Lipid peroxidation, t-SOD and CAT enzyme activities in adult animals of F2 generation

In adult animals of the F2 generation, similar effects were induced by the experimental diet in the two regions analyzed. As shown in the **Figure** 1A, evidence of lipid peroxidation, assessed by a significant increase in TBARS levels, was detected in the SN and CS of the EF2A group (0.82 ± 0.1 and 0.9 ± 0.18 nmol MDA / mg protein, respectively) in comparison with the control group (0.4 ± 0.03 and 0.35 ± 0.02 nmol MDA / mg protein, $p < 0.05$). The magnitude of lipid peroxidation induced by the experimental condition in the SN is about 65% 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 CS of the EF2A 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 the EF2A and CF2A groups, respectively) (**Figure 1B).** On the other hand, the CAT activity was significantly

reduced in the SN (2.38 ± 0.21 nmol/min/mg protein) and CS (3.40 ± 0.60 nmol/min/mg protein) of the EF2A group compared to their respective control groups (5.20 ± 1.11 and 9.58 ± 1.37 nmol/min/mg protein for SN and CS respectively; $p < 0.001$; **Figure 1C**).

Please, insert Figure 1 around here

Nitric oxide levels in the SN and CS of young and adult animals

Figure 2 shows the results obtained in the SN and CS homogenates of F2 young and adult animals. In the SN, no intergroup difference in the nitrite levels was observed either in young or in adult animals. However, in the CS of both young and adult EF2 groups, nitrite levels were respectively 30% and 1.8-fold higher than those of the control group ($p < 0.001$). It is worth noting that in the CS of the control group, nitrite levels were estimated as about 3-fold higher than those estimated in the SN of the same group.

Please, insert Figure 2 around here

Number of TH positive neurons at the middle level of SNrm and SNcv in adult animals

Panel A in Figure 3 shows low magnification images of representative parasagittal sections immunoreactive for TH through SNrm and SNcv in animals of CF2A and EF2A groups. As can be observed, TH immunoreactivity is less intense in both SNrm and SNcv of the EF2A animals. On average, the numbers of TH-immunoreactive cells in the middle level of SNrm and SNcv of the control group were 535 ± 106 and 236 ± 51,9 respectively.. A comparative analysis between the two groups showed that in the SNrm and SNcv the number of TH-immunoreactive cells in adult rats fed the deficient diet was, respectively, 278 ± 48.3 and 142 ± 25.9 which corresponds to ~50 and 40% fewer cells than in those fed on the control diet. (Fig 3B)

Please, insert Figure 3 around here

Effects of the dietary treatment on SN dopaminergic cell soma size in adult animals

The long term dietary treatment with the experimental diet being used until the adult stage resulted in lower dopaminergic cell size both in the SNrm (median = 188.7 μm^2 versus 243 μm^2 in the control) and the SNcv (248.1 μm^2 versus 296,2 μm^2 in the control) using the Kruskal–Wallis ANOVA Ranks test, followed by the Dunn's test, $P < 0.001$ for both regions (Fig. 4).

Please, insert Figure 4 around here

Number of BDNF positive neurons at the middle level of SNrm and SNcv in adult animals

Figures 5A and B illustrate low magnification images of representative brain parasagittal sections throughout the SN in animals of CF2A and EF2A groups. As can be seen, BDNF-immunoreactivity is widely distributed throughout the entire extension of this nucleus. Fig. 5B, 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 any heterogeneous SN cell population, with respect to soma size and shape. Quantitative analysis of BDNF expressing cells performed with 5 animals per group at the middle level of SN (in 3 parasagittal sections per animal) showed that the number of SN BDNF-immunoreactive cells in rats fed the deficient diet was 20% lower (1,009 ± 156.8 cells; $p < 0.05$) than in those fed the control diet (1,292 ± 156.6 cells) (Fig. 5G).

Please, insert Figure 5 around here

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 EF2 adult animals are shown in **Figure 6**. As can be seen, single (Figs. 6A and 6B, B"). or double labeled cells Figs. 6C.D,E) are detected in the SN of both groups Nevertheless, several remaining TH-positive cells in the EF2A animal are also positive for BDNF, while in others, the presence of this neurotrophin was not visualized.

Please, insert Figure 6 around here

Discussion

The present study investigated whether the essential fatty acids dietary restriction 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 (Cardoso et al., 2012). Moreover, it was hypothesized that a long term DHA deficiency until adult stage could reduce the CS resilience observed in young animals, affecting it's redox balance. The results partially corroborated our hypothesis, demonstrating that NO production and t-SOD activity in the SN and CS were differentially affected by this type of nutritional insult. In addition, the reduced number of

BDNF positive neurons in the SN of omega 3 deficient animals reinforces the partial involvement of this neurotrophin for DHA-induced neuroprotection.

As has been previously reported for young animals (Borba et al., 2010; Passos et al., 2012), the long term treatment with an EFA deficient diet until adult stage was able to induce systemic effects on the rat somatic growth as shown by the reduced body weight detected in the experimental animals. This data is consistent with the effect of diets containing coconut oils as the only source of lipids in reducing body weight gain and such reduction seems to be independent of essential fatty acid deficiency (Hargrave et al., 2005). On the other hand, the negative repercussion on the brain weight observed in young animals (Passos et al., 2012) was not detected in the experimental group at the adult stage, suggesting compensatory mechanisms during brain maturation. Previous studies adopting a dietary deficiency specific for α-linolenic fatty acid for two or three generations also have not reported any difference in the brain weight between control and n-3 deficient groups at adulthood (Ahmad et al., 2002b).

The increasing DHA deficiency in both SN and CS of adult EF2 group (~65% reduction relative to control) was able to reduce the resilience of CS to oxidative insult, previously observed in young animals (Cardoso et al., 2012). Moreover, increased lipid peroxidation levels in the SN (~2 fold, compared to control) also affected the dopaminergic neurons located in the caudo-ventro-lateral region. Nevertheless, it should be noted that similar levels of DHA depletion in both SN and CS induced distinct mechanisms underlying the oxidative stress and neuronal cell loss herein described, especially those involving NO production.

Nitrergic terminals have been reported to make synaptic contacts with both substantia nigra dopaminergic neurons and their terminal areas such as the CS (West and Tseng, 2011). An interesting piece of information obtained in the present study was that, in contrast to our initial hypothesis, the EFA dietary restriction over two generations did not modify NO levels in the SN, neither at the young nor the adult stage, indicating that in this nucleus, modifications in the synthesis or release of this bioactive substance 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 (Calabrese et al., 2007; Pierucci et al., 2011; West and Tseng, 2011). While in physiological concentrations NO is able to prevent apoptotic events induced by hypoxia (Singh and Dikshit, 2007), in some neurodegenerative diseases, such as Parkinson´s disease, a high concentration of NO leads to dopaminergic cell death (SINGH DIKSHIT, 2007). An increased number of nNOS expressing neurons was 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. However, an increase in nNOS expression was not involved in nigral cell degeneration, suggesting that NO could have a protective rather than a neurotoxic role under that condition (Gonzalez-Hernandez et al., 1997). A modulatory action of DHA on NO production has been discussed, indicating that the dietary supplementation of this essential fatty acid can reduce the activity of NOS in some brain regions (Sarsilmaz et al., 2003**).** DHA also reduces NO production in reactive microglia as one potential mechanism involved in its anti-inflammatory action (Lu et al., 2010). Our present findings, showing that DHA depletion did not change NO contents in the SN under conditions of oxidative stress, deserve future studies, especially to investigate whether this dietary treatment could modify microglia reactivity in this nucleus.

On the other hand, in the CS, raised NO levels in EF2 young and adult animals occurred with different magnitude and conditions of homeostatic response. While a 30rise in NO contents was detected in young animals, where the t-SOD enzyme was reactive and LP was not observed (Cardoso et al.,2012), an expressive elevation of ~1.8 fold was found in adult animals in a context with reduced t-SOD and CAT activities and the LP was twice as high as in the control condition. NO has been indicated as an important modulator of the basal ganglia circuit, exerting important functions in movement control or in pathophysiological conditions (Pearuci et al., 2011; West and 2011). In the CS, nitrergic interneurons are involved in the corticoestriatal glutamatergic excitability and NO synthesis can be modulated by dopamine via its receptor subtypes D1 and D2 (West and Tseng 2011). An increase in the nNOS cell density or NADPH-diaphorase activity in the CS after chronic nigro-striatal deafferentation has been reported (Gomes and Del Bel, 2003; Sancesario et al., 2004). On the other hand, under conditions of oxidative stress induced by 6-OHDA injection into the CS, pretreatment with a NO donor worsened the dopamine cell degeneration in the SN (Di Mateo et al., 2009). Consistent with this evidence, a nitric oxide synthase inhibitor decreased 6-OHDA effects on dopaminergic neurons in the rat nigrostriatal pathway (Gomes et al., 2008). Recent study has also indicated that DHA deficiency induces microglia activation in the CS (Kuperstein et al., 2009). Considering that induced NOS (iNOS) activity can be triggered in reactive microglia under neurodegenerative conditions, we cannot discard the possibility that the high NO concentration observed in the CS of our adult experimental animals could be a result of a neuroinflammation induced by DHA depletion in this nucleus. It is well established that an excessive amount of NO can lead to the formation of peroxynitrite (ONOO-) and other reactive nitrogenous species (RNS) nitrate tyrosines of proteins to form 3-nitrotyrosine, leading to cell death (Bishop et al., 2009). Thus, it is possible that the expressive reduction in

both t-SOD and catalase enzymatic activities observed in the CS of our omega-3 deficient animals can be partially due to this type of deleterious effect on these proteins.

Gomes et al., (2008) also demonstrated that dopaminergic cells located in the ventral region of SN were more affected by 6-OHDA- induced oxidative stress in the CS than in other SN dopaminergic cell populations. In the present study, dopamine cell loss in the caudo-ventro-lateral region was detected only in adult animals, when oxidative stress was also seen in the CS. Our findings about these conditions of DHA deficiency reinforce previous data, indicating vulnerability of this dopamine cell population to conditions of reduced redox balance (Rodrigues et al., 2001; Gomes et al., (2008). A distinct neurochemical profile related to higher levels of plasma membrane in the dopamine transporter (Uhl et al., 1994; Gonzalez-Hernandez et al., 2004), increased expression of genes encoding pro-inflammatory cytokines and decreased expression of several glutathione-related genes (Duke et al., 2007), have been described for this SN dopaminergic cell population. Thus, it is possible that the oxidative stress reported in the SN of F2 young animals submitted to our experimental dietary model may not be the main mechanism involved in the loss of dopaminergic cells located at the SNrm (Cardoso et al., 2012). These cells are usually considered more resistant to different types of insults, including oxidative stress (Barroso-Chinea et al., 2005). Indeed, preliminary results of our group support this idea, suggesting that loss of dopaminergic cells in the SNrm has already occurred in adult animals of the EF1 generation (unpublished data). In these animals, there were no signs of LP resulted from EFA dietary restriction (Cardoso et al., 2012).

We hypothesized that another potential mechanism involved in SN neurodegeneration induced by DHA deficiency could be a reduced number of BDNF positive neurons in the SN of adult EF2 animals. BDNF is believed to act as a paracrine/autocrine neurotrophic factor for dopaminergic and non dopaminergic neurons of nigrostriatal system (Hyman et al; 1991, 1994; Stahl et al., 2011). Evidence in several post mortem studies in human beings have indicated that loss of BDNF-expressing neurons in the SN may compromise their surviving neighbors, reducing the amount of their BDNF mRNA (Howells et al., 2000). In the mouse SN, BDNF is present in dopaminergic, GABAergic and nNOS positive neurons. In addition, this neurothophin was also observed in GFAP-positive astrocytes (Abe et al., 2010). A positive influence of DHA on BDNF levels and/or activity of its receptor TrkB has been reported in the hippocampus, cerebral cortex (Bousquet et al., 2009; Vines et al., 2012) and spinal cord (Ying et al., 2012) as well as in the CS (Sarsilmaz et al., 2003). To our knowledge no previous study has investigated the repercussion of DHA deficiency on BDNF expression

in the SN. Increased content of other neurotrophins such as GDNF and neurturin in this nucleus has been observed upon DHA supplementation, reducing dopaminergic cell death induced by MPTP, (Tanriover et al., 2010). In the mouse, DHA supplementation also raised BDNF mRNA expression in the CS and BDNF protein levels in the cerebral cortex (Bousquet et al., 2009). 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 cerebral cortex (Rapoport et al., 2007; Rao et al., 2007) and mouse CS (Miyazawa et al., 2010).

In the present work, we demonstrated that the number of BDNF positive cells was 20% lower in the SN of adult EF2 group, compared to the control. While this at first appears to be consistent with loss of the nigral dopaminergic neurons, double labeling experiments demonstrated that several surviving TH-immunoreactive cells also expressed BDNF in their soma and dendrite processes while in other remaining TH-positive cells, BDNF immunoreactivity was not detected. It has been demonstrated that BDNF expression in the midbrain slice culture can be enhanced by agonists of retinoid acid receptors (RAR) whose oral administration prevents dopaminergic cell loss induced by neuroinflammation in the SN (Katsuki et al., 2009). Adopting the same type of organotypic midbrain slice culture, but in the absence of inflammatory stimuli, Kurauchi et al., (2011) also showed that RAR-induced BDNF upregulation depends on NO signaling in the SN dopaminergic neurons. DHA as well as AA are ligands for the retinoid X receptor (Lengqvist et al., 2004) which, together with RAR, play diverse roles in brain development, including morphological differentiation of dopaminergic neurons (Castro et al., 2001). Some studies have also shown that EFA deficiency can modify the expression of genes involved in a number of cellular mechanisms, and many of these are downstream targets of RAR-RXR signaling (Kitajka et al., 2002). Another point to be discussed is the fact that DHA supplementation can reduce the loss of the transcription factor Nurr1 in the SN under conditions of MPTP-mediated oxidative stress (Bousquet et al., 2009). It has been reported that BDNF is a direct target gene of Nurr1 (Volpicelli et al., 2007). Taking into consideration that Nurr1 is involved in the genesis, development and function of dopaminergic cells (Jankovic et al. 2005), it is possible to speculate that the reduced number of BDNF–expressing neurons and the lower values of cell body area observed in two SN dopaminergic cell populations of EF2A animals, can be a consequence of diverse cellular and molecular mechanisms which can be impaired under conditions of chronic DHA deficiency.In conclusion, our present findings demonstrate for the first time that there is a differential effect of EFA dietary restriction on NO production in the nigrostriatal system. Moreover, the data corroborated our initial hypothesis that an increasing

DHA depletion could worsen the dopamine cell loss, reducing CS resilience, the number of SN BDNF-expressing neurons, and anti-oxidant resources. Nevertheless, distinct mechanisms of oxidative stress induced by this type of nutritional insult were detected in the SN and CS of adult rats. Taken together, the results reinforce the idea that EFA dietary restriction during brain development and maturation can impair some homeostatic mechanisms which alone or in combination modify the degeneration profile of midbrain dopaminergic cells and the brain's competence to maintain suitable resilience under challenging conditions during adult life.

Conflict of interest

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

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LEGENDS

Figure 1. Thiobarbituric acid-reactant substances (TBARS) levels **(A),** total superoxide dismutase (t-SOD) activities **(B)** and catalase (CAT) activities **(C)** in the Substantia Nigra and Corpus Striatum from adult rats fed essential fatty acid restricted diet over two generations and respective controls (n = 12 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 EF2 groups.

Figure 2: Nitrite concentration as indicator of Nitric oxide production in the substantia nigra (SN) and corpus striatum (ST) from adult rats fed essential fatty acid restricted diet over two generations and respective controls (n = 12 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 EF2 groups. Nitrite concentration was determined by the Griess reagent.

Figure 3. Panel A: Representative photomicrographs of TH-immunoreactive parasagittal sections at the middle level of Substantia Nigra from adult rats fed control or experimental diet for two generations showing dopaminergic cells in the substantia nigra rostro-dorso-medial (SNrm) (a,c) and caudo-ventro-lateral (SNcv) (b,d) (bar = 30 μm). **B:** Contrast indices for average number of TH-immunoreactive cells obtained in three parasagittal sections at the middle level of SNrm and SNcv from adult rats fed control or experimental diet. Negative values in both regions indicate the deleterious effects of chronic EFA dietary restriction on dopaminergic cell distribution Note that the SNrm is more affected than the SNcv. The contrast index is defined by the ratio indicated in the Y-axis, where C and E correspond to the values obtained for control and experimental groups, respectively. All mean values and significances are described in the text.

Figure 4. Comparative dopaminergic cell area in the SNrm (A) and SNcv (B) of control and experimental groups showing the median, maximum and minimum values of the predominant classes of soma size among the regions and groups (N=300 cells per region). (C) The experimental condition resulted in lesser average soma size in both the SNrm (~20%) and SNcv (~15%) when compared to the control condition (P < 0.01, Kruskal-Wallis test followed by the Dunn´s test). #, difference between regions; §, difference between groups.

Figure 5. A. Representative photomicrographs of BDNF-immunoreactive parasagittal sections at the middle level of Substantia Nigra from adult rats fed control or experimental diet. Low magnification images of CF2 (A) and EF2 (D) animals showing the evenly distribution of BDNF-expresing cells into the cytoarchitetonic limits of substantia (bar = 250 μm). High magnification images of showing distribuition of BDNF positive cells in the SNrm and SNcv from CF2 (B and C) and EF2 (E and F) animals (bar = 30 μm). **B.** Average number of BDNF positive neurons in the SN **of** CF2 and EF2 groups. Data were obtained in three parasagittal sections at the middle level of SNrm and SNcv and express mean ± SD. **P < 0.001 compared to control group.

Figure 6. Photographs of epifluorescence microscopy showing SN sections double-labeled for BDNF stained with DAB followed by TH visualized with DyLight-conjugated 488-IgG **.** Examples of single and adjacent BDNF (brown) or TH (green) immunoreactive cells are

shown for rats fed control (A) or experimental (B) diets. Double labeled cells are seen either in the control (C and D) or in experimental animals (E and F). Scale bar = 20μm

TABELAS

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[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

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

Fatty acids	Control diet	Experimental 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

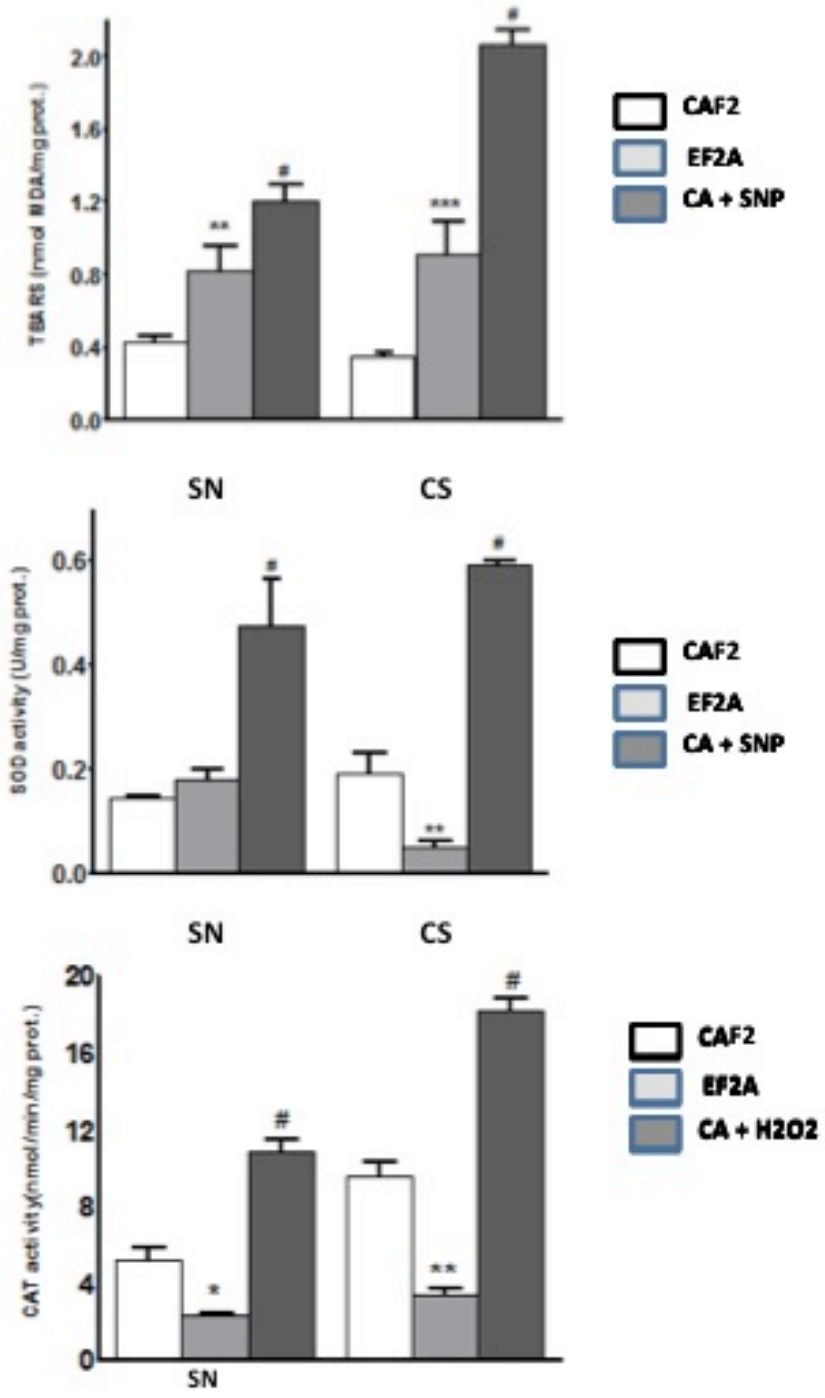
nd, not detected. Bold values indicate $p < 0.001$.

Table 3 Body and Brain weights

	Body weight	Brain weight	Brain weight/body weight ratio
CF2 Y	79.65 ± 14.87 ; (*n* = 33)	1.69 ± 0,06; (n=08)	0,021 ± 0,004; (n=08)
EF2 Y	71.91±10.09*; (*n* = 40)	1,48 ± 0,123 ***;(n=10)	0,020 ± 0,010; (n=10)
CF2 A	385,46 ± 41,75; (n=15)	2,05 ± 0,18; (n=06)	0,0050 ± 0,004 ; (n=06)
EF2 A	338,28 ± 36,68 **; (n=2	1,91 ± 0,12; (n=09)	0,0056 ± 0,005 ; (n=09)

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

FIGURAS



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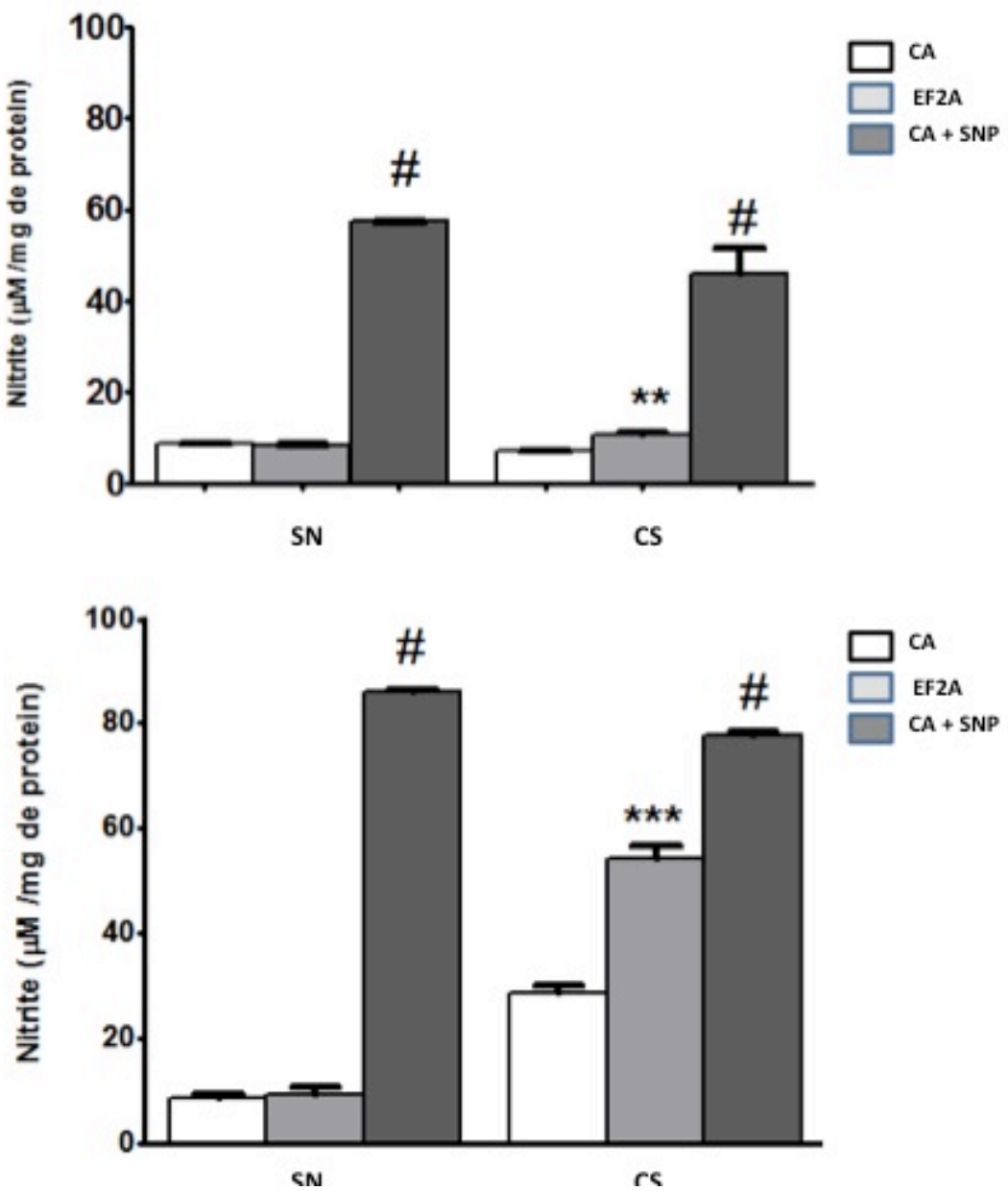

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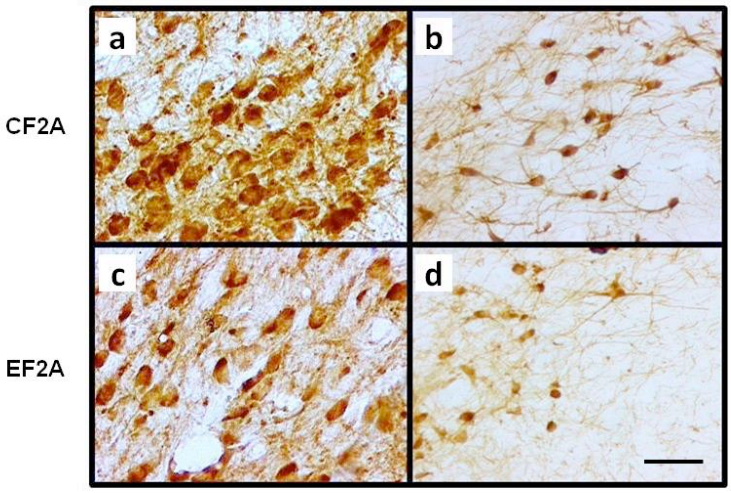
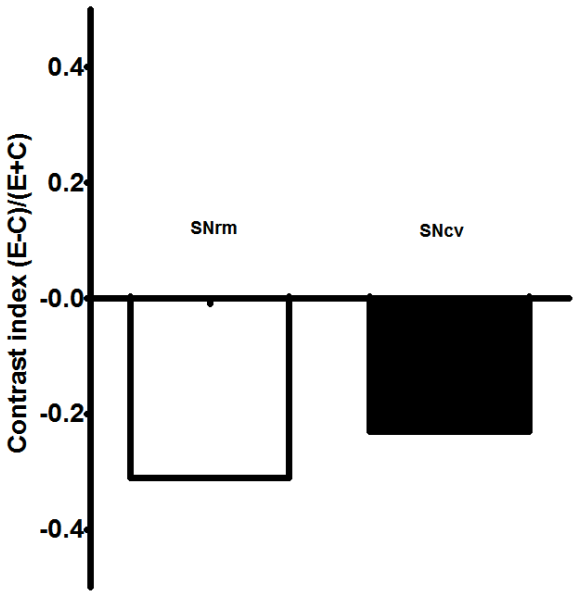
PANEL A



PANEL B



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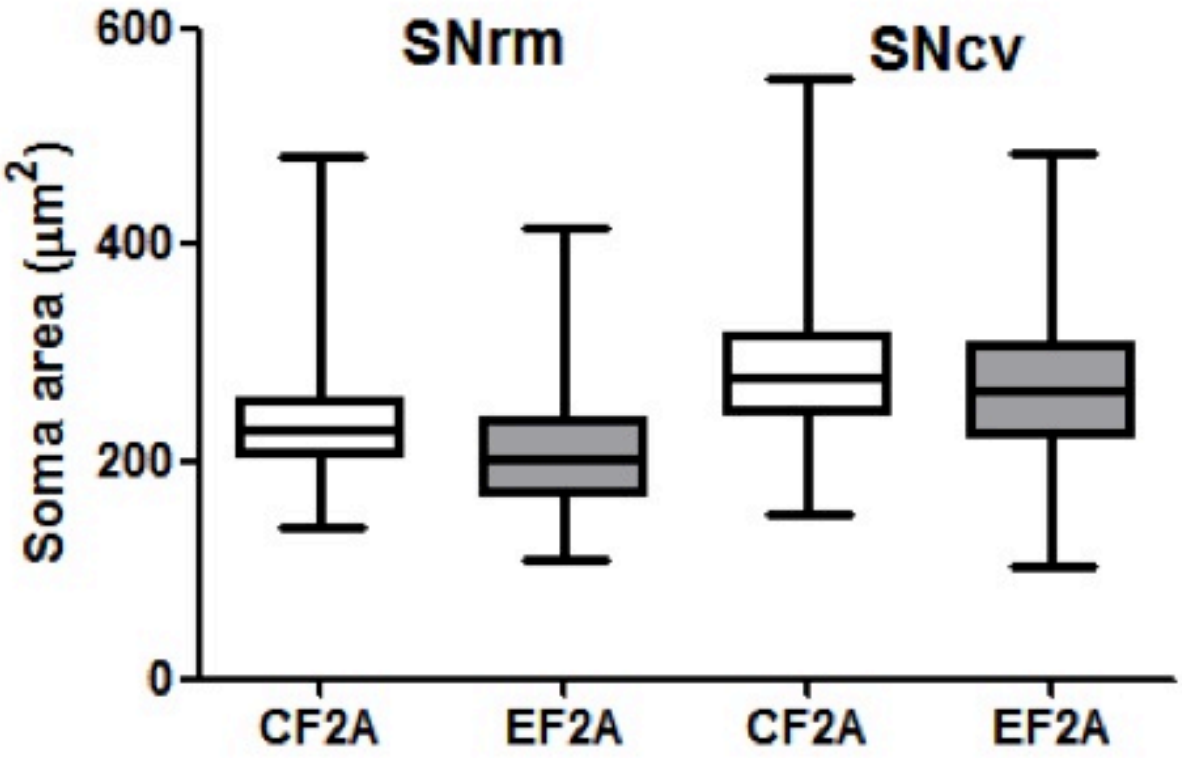

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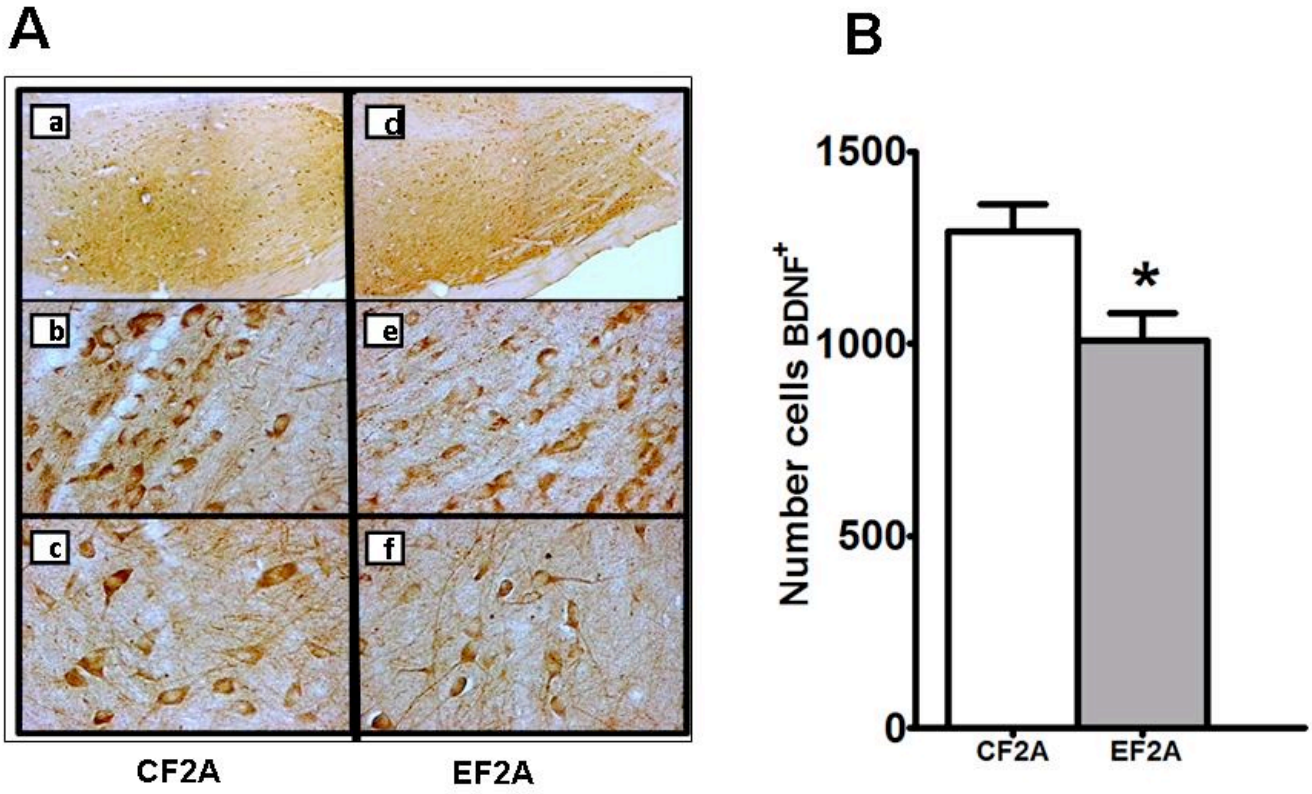

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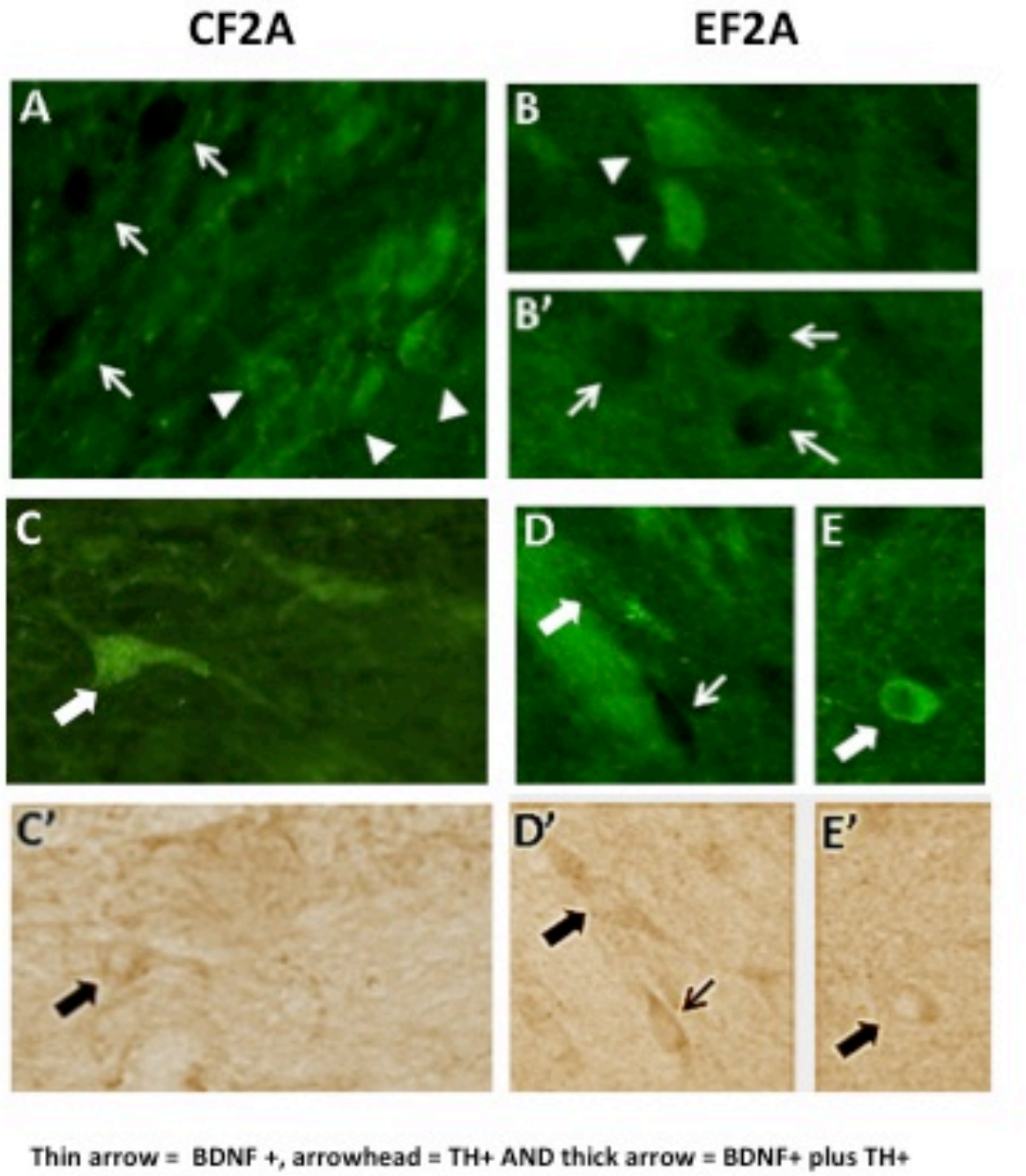

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9.2 Parecer do Comitê de Ética em Experimentação Animal da UFPE

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Ofício nº 44/06

Recife, 01 de agosto de 2006

Da Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Profa. Belmira Lara da Silveira Andrade da Costa**
Departamento de Fisiologia e Farmacologia – UFPE
Processo nº 009428/2006-33

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram seu projeto de pesquisa intitulado **"LIPÍDIOS DA DIETA E DESENVOLVIMENTO DO SISTEMA DOPAMINÉRGICO: IMPACTO SOBRE A ESTRUTURA E FUNÇÃO CEREBRAL".**

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 CEEA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

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

Atenciosamente,

Prof. Silene Carneiro do Nascimento
UFPE
Presidente CEEA

CCB: Integrar para desenvolver