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DEPARTAMENTO DE FISIOLOGIA E FAMACOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

TESE DE DOUTORADO

MECANISMOS NEUROQUÍMICOS ENVOLVIDOS NA NEURODEGENERAÇÃO DA *SUBSTANTIA NIGRA* INDUZIDA PELA RESTRIÇÃO DIETÉTICA EM ÁCIDOS GRAXOS ESSENCIAIS

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RECIFE-PE
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Pelo sopro de Deus se dá a geada, e as largas águas se congelam. Também de umidade carrega as densas nuvens, nuvens que espargem os relâmpagos. Então elas, segundo o rumo que Ele dá, se espalham para uma e outra direção, para fazerem tudo o que lhes ordena sobre a redondeza da terra.

Jó 37, 10-12.

"No momento em que uma célula masculina microscópica e serpenteante se encaminha para a célula- ovo -muito maior e liga se à ela, um ser humano começa a existir , e a nutrição tem início. Este período de desenvolvimento, quando as coisas podem ser definitivamente 'certas' ou 'erradas', é de vital importância, e a nutrição pode exercer uma profunda influência que se expande por toda a vida". (Roger Willians)

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Dedico este trabalho à minha amada família

SUMÁRIO

RESUMO

Os ácidos graxos essenciais (AGEs) têm sido indicados como potenciais agentes preventivos e terapêuticos em uma grande variedade de doenças neurodegenerativas assim como indispensáveis para o desenvolvimento cerebral. O principal objetivo deste trabalho foi investigar os mecanismos relacionados com a perda de neurônios dopaminérgicos induzida pela deficiência crônica em AGEs previamente detectada na substância negra (SN) de ratos *Wistar* jovens (J), estendendo a análise também a animais adultos (A). Para isso, foram utilizadas dietas balanceadas e que diferiram apenas na fonte lipídica, sendo óleo de soja para os grupos controles (C) e óleo de coco para os grupos experimentais (E). As dietas foram fornecidas às mães a partir do acasalamento e mantidas por uma (F1) ou duas (F2) gerações. Marcadores de insulto oxidativo: lipoperoxidação (LP), atividade das enzimas superóxido dismutase total (SOD-t), catalase (CAT) na SN e corpo estriado (CE) foram avaliados em animais AF1 e em JF2 e AF2. Indicadores de neurodegeneração na SN e CE destes animais foram avaliados utilizando a técnica de marcação com o fluoróforo, Fluoro Jade C. Análise quantitativa do tamanho e nº de neurônios dopaminérgicos e da distribuição de células imunorreativas ao fator neurotrófico derivado do cérebro (BDNF) foi realizada em animais AF2. Os níveis de nitrito, como indicador da produção de óxido nítrico na SN e CE, foram analisados em animais JF2 e AF2. A dieta experimental reduziu em ~28%, ~50% e ~60% os níveis de ácido docosahexaenóico (DHA) na SN dos grupos experimentais AF1, JF2 e AF2 respectivamente, comparado aos seus controles. Nos animais EAF1 um aumento em ~17% e ~45% na atividade da SOD-t foi observado na SN e CE comparado ao grupo controle, o evitou níveis danosos de lipoperoxidação. Por outro lado, um aumento nos níveis de lipoperoxidação (~34%) foi detectado na SN de animais EJF2, acompanhados de não reatividade da SOD-t e de uma redução em 4,8 vezes na atividade da CAT. Sinais de neurodegeneração foram evidenciados em neurônios dopaminérgicos e não dopaminérgicos da SN do grupo EJF2. No CE, o aumento da LP em ~39% foi acompanhado de redução em 3,8 vezes e 2,8 vezes da atividade da SOD-t e CAT, respectivamente, só foram observados nos animais do grupo EAF2. A dieta experimental não alterou os níveis de nitrito na SN, mas aumentou de forma significativa estes níveis no CE de animais jovens (30%) e adultos (1,8 vezes). A deficiência crônica em DHA até a idade adulta comprometeu o crescimento do corpo celular e aumentou a perda de neurônios dopaminérgicas na SN rostro-dorso-medial (~35%) afetando também aqueles localizados na região caudo-ventro-lateral deste núcleo. Uma redução de ~22% no número de células $BDNF^{+}$ foi também observada na SN. Os resultados mostram que a restrição dietética em AGEs por duas gerações até a idade adulta é capaz de induzir lipoperoxidação na SN e CE devido a comprometimento na atividade das enzimas anti-oxidantes, perda de células $BDNF^{+}$ na SN e aumentados níveis de óxido nítrico no CE. Tidos em conjunto, tais mecanismos podem estar atuando de forma sinérgica na degeneração de neurônios dopaminérgicos induzida pela deficiência em DHA.

Palavras Chaves: Ácidos graxos essenciais, Má-nutrição, *Substantia nigra*, *Corpus estriatum*, Peroxidação lipídica, Superóxido dismutase, Catalase, Óxido nítrico e Neurodegeneração.

ABSTRACT

Essential fatty acids (EFAs) have been suggested as potential therapeutic and preventive agents in a variety of neurodegenerative diseases as well as essential for brain development. The main objective of this study was to investigate the mechanisms associated with loss of dopaminergic neurons induced by chronic deficiency in AGEs previously detected in the substantia nigra (SN) of young Wistar rats (Y), extending the analysis also adult animals (A). For this, we used balanced diets and differed only in lipid source, and soybean oil for the control groups (C) and coconut oil for the experimental groups (E). Diets were fed to mothers from mating and maintained by one (F1) or two (F2) generation. Markers of oxidative insult: lipid peroxidation (LP), activity of superoxide dismutase total (t-SOD), catalase (CAT) in the *substantia nigra* (SN) and *corpus striatum* (CE) were evaluated in animals AF1 and AF2 and YF2. Indicators of neurodegeneration in these animals CE and SN were assessed using the technique of labeling with the fluorophore, Fluoro Jade C. Quantitative analysis of the size and number of dopaminergic neurons and the distribution of immunoreactive cells to brain-derived neurotrophic factor (BDNF) was performed in animals AF2 *substantia nigra*. The nitrite levels, as an indication of nitric oxide production in CE and SN were analyzed in animals YF2 and AF2. The experimental diet reduced by ~ 28%, ~ 50% and ~ 65% levels of docosahexaenoic acid (DHA) in the SN of the experimental groups AF1, AF2 and YF2 respectively, compared to their controls. In animals EAF1 increased by ~ 17% and ~ 45% in t-SOD activity was observed in SN and CE compared to the control group, avoided harmful levels of lipid peroxidation. Moreover, an increase in lipid peroxidation levels (~34%) was detected in the SN of animals EYF2, accompanied by non-reactivity of t-SOD and a reduction by 4.8 fold in CAT activity. Signs of neurodegeneration were observed in dopaminergic neurons of SN dopaminergic and non EYF2 group. In CE, the increase in LP (~ 39%) was accompanied by reduction 3.8 and 2.8 fold of the SOD activity and CAT, respectively, were observed only in animals EAF2. The experimental diet did not alter the levels of nitrite in the SN, but significantly increased these levels in the CE of young animals (~30%) and adults (1.8 fold). A chronic deficiency in DHA into adulthood affected the growth of the cell body and increased dopaminergic neuronal loss in the SN rostro-medial dorsal (~35%) also affecting those located caudal-ventrolateral nucleus of this. An ~ 22% reduction in cell number $BDNF^{+}$ was also observed in the SN. The results show that dietary restriction in AGEs for two generations until adulthood is able to induce lipid peroxidation in SN and CE due to impairment in the activity of anti-oxidant enzymes, cell loss $BDNF^{+}$ in SN and increased levels of nitric oxide in CE. Taken together, these mechanisms may be acting synergistically in the degeneration of dopaminergic neurons induced by DHA deficiency.

Key-words: Essential fatty acids, malnutrition, *substantia nigra*, *corpus striatum*, lipid peroxidation, superoxide dismutase, catalase, nitric oxide and neurodegeneration.

LISTA DE ABREVIATURAS

AGEs- Ácidos graxos essenciais
AGPI-CL- Ácidos graxos polinsaturados de cadeia longa
ALA- ácido alfa-linolênico
BDNF-Fator Neurotrófico Derivado do Cérebro
CAT – Catalase
CREB – proteína ligante ao elemento de resposta ao AMPc
DNA – Ácido Desoxirribonucléico
DHA- ácido docosahexahenóico
DP - Doença de Parkinson
DPA- ácido docopentaenóico
EPA- ácido eicosapentaenoico
ERK – proteína cinase regulada por sinais extracelulares
ERN- Espécies Reativas de nitrogênio
ERO – Espécies Reativas ao Oxigênio
GABA – Ácido gama aminobutírico
GDNF-Fator Neurotrófico Derivado da Glia
GHS - Glutationa
GMPc – 3' 5' monofosfato cíclico de guanosina
GPe - Globo Pálido externo
GPi - Globo Pálido interno
GPx – Glutationa peroxidase
H_2O_2 – Peróxido de Hidrogênio
LA- ácido linoleico
MDA – Malondialdeído
NADPH – Nicotinamida adenina dinucleotídeo fofosfato
NO- Óxido Nítrico
NOS – óxido nítrico sintase
NPD1- Neuroprotectina D1
$O2^{-}$ - ânion superóxido
O2 – Oxigênio molecular
OH^{-} – Radical Hidroxil
$ONOO^{-}$ - peroxinitrito
PLAc – fosfolipase C
PEM- Malnutrition protein energy.
DPC- Desnutrição protéico-calórica
RNAm – Ácido Ribonucléico Mensageiro
SNrm – Substancia Negra rostro-dorso-medial
SNcv – Substancia Negra caudo-ventro-lateral
SNC-Sistema Nervoso Central
SOD-t – Superóxido Dismutase total
TBARS – Espécies Reativas ao Ácido Tiobarbitúrico
TrKB- receptor tirosina cinase B
ω 3- Ácidos graxos da série ômega-3
ω 6- Ácidos graxos da série ômega-6

LISTA DE FIGURAS DA FUNDAMENTAÇÃO TEÓRICA

1. INTRODUÇÃO

Os ácidos graxos (AG) saturados, monoinsaturados e poli-insaturados de cadeia longa desempenham importantes funções na estrutura e atividade metabólica das membranas celulares. O sistema nervoso, em especial, deve conter níveis equilibrados destes ácidos graxos. Dentre a diversidade deles existem os que o organismo de mamíferos não é capaz de sintetizar, sendo estes denominados de ácidos graxos essenciais, pertencentes às famílias ω-3 e ω-6, cujos precursores são o ácido α-linolênico (C18:3, n-3) e o ácido linoléico (C18:2, n-6) respectivamente.

Durante a vida intrauterina e período de aleitamento os ácidos α-linolênico, linoléico e seus respectivos derivados, o ácido docosahexaenóico (DHA) e ácido araquidônico (AA) são adquiridos através da transferência materno-placentária e leite materno. Após o desmame, os mesmos são obtidos unicamente por uma alimentação balanceada em macronutrientes ao longo da vida. As devidas incorporações do DHA e AA na estrutura da membrana plasmática confere melhor desempenho das funções cerebrais e visuais, de modo que têm sido indicados como potenciais agentes neuroprotetores, favorecendo o crescimento neuronal, transdução de sinais, excitabilidade, expressão de genes e a atuação de fatores neurotróficos que regulam a sobrevivência e diferenciação celular. (BOURRE, 2006; BOUSQUET, 2009; BRADBURRY, 2011 ; QUERQUES, 2011).

Os AGEs são considerados como potenciais agentes terapêuticos em uma grande variedade de doenças neurológicas e psiquiátricas (MACNAMARA & CARLSON; 2006; BAZAN, 2011). Nas últimas décadas, vários estudos têm destacado a importância da ingestão dos AGEs para melhorar o prognóstico de doenças degenerativas, como a doença de Parkinson, Alzheimer, esclerose múltipla, entre outras. A investigação das propriedades desses macronutrientes terapêuticos tem sido de grande relevância e aplicabilidade na clínica e em modelos experimentais devido às suas potenciais ações anti-inflamatórias, antioxidantes e anti-apoptóticas. Por exemplo, estudos revelaram que um baixo teor de α-linolênico dietético está relacionado com baixos índices neurológicos e cognitivos, déficits de memória, alterações de humor e comprometimento da atividade locomotora (YEHUDA et al., 1997; GOMEZ-PINILLA et al., 2008).

Recentes evidências obtidas em nosso laboratório, utilizando um modelo de restrição dietética em AGEs por duas gerações revelaram uma vulnerabilidade diferenciada entre duas subpopulações de neurônios dopaminérgicos da *substantia nigra* (SN) a repercussão dessa dieta. Tal tratamento reduziu em cerca de 20% o número de neurônios dopaminérgicos da região rostro-dorso-medial da SN de animais jovens, mas não da região caudo-ventro-lateral. Além disto, afetou o crescimento de todos os neurônios

dopaminérgicos deste núcleo e reduziu os níveis proteicos da enzima tirosina hidroxilase (PASSOS et al., 2012). Considerando que as subpopulações celulares na SN apresentam peculiaridades bioquímicas as quais conferem diferentes vulnerabilidades a lesões oxidativas, quando comparadas entre si ou com outras regiões do cérebro, o presente estudo foi conduzido para testar a hipótese de que aumentados níveis de estresse oxidativo e reduzidos níveis de neurotrofinas podem ser potenciais mecanismos envolvidos na perda de células dopaminérgicas da SN rostro-dorso-medial em animais jovens, previamente descritas. Para testar se a depleção progressiva nos níveis de DHA, induzida pela restrição dietética crônica em AGES, poderia provocar perda de células dopaminérgicas da região caudo-ventro-lateral e comprometer outros tipos de neurônios na *substantia nigra*, analisamos também os animais adultos da segunda geração.

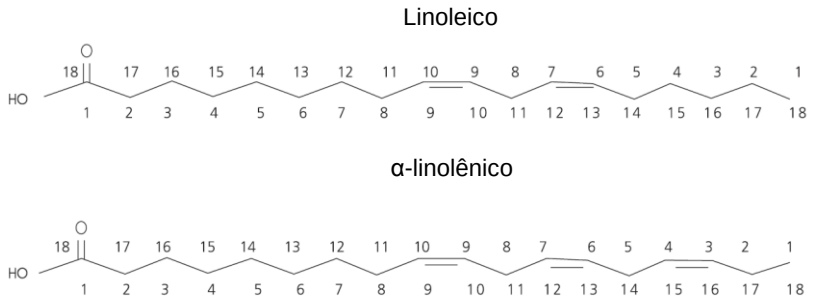
2. FUNDAMENTAÇÃO TEÓRICA

2.1.Os Ácidos Graxos Essenciais

No final da década de 20, alguns pesquisadores já constatavam a importância dos AGEs, ao observarem sinais de alterações dérmicas em ratos e distúrbios neurológicos e visuais em humanos que tinham sido submetidos a restrição de gorduras em suas dietas (BURR, 1929). Desde então os AGEs vêm sendo estudados, porém, somente na década de 70, houve uma maior evolução nas pesquisas envolvendo esses tipos de ácidos graxos e a importância de níveis equilibrados dos mesmos na dieta (GROSSMAN, 1975; BRADBURRY, 2011).

Os AGEs, uma vez consumidos, podem ser alongados e dessaturados enzimaticamente, convertendo-se em ácidos graxos poli-insaturados de cadeia longa (AGPI-CL) de pelo menos 20 ou 22 carbonos, como ilustrado na Figura 1.

A



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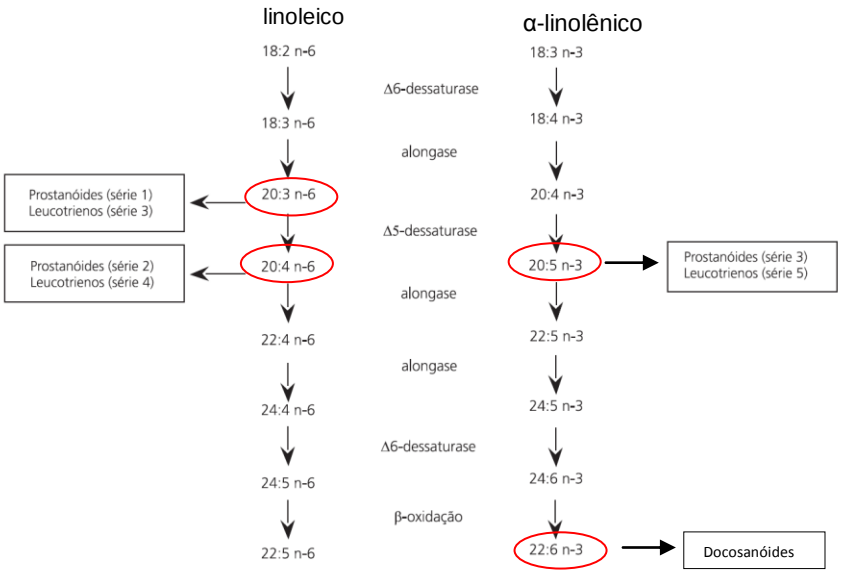


Figura 1. Estrutura dos ácidos graxos essenciais, linoleico e α-linolênico (A); Metabolismo dos ácidos linoleico e α-linolênico envolvido na síntese dos AGPI-CL (B). Figura extraída de INNIS, 2003.

Do grupo dos AGEs, o principal AGPI-CL representante da série ou família ômega 6 é o ácido araquidônico (AA; C20:4,n-6) e da família ômega 3, os ácidos eicosapentanóico (EPA 20:5, n-3) e docosahexanóico (DHA; C22:6,n-3). As enzimas participantes do metabolismo dos mesmos, as alongases e dessaturases, competem por substratos oferecidos pelas duas famílias (SALEM, 1999). A concorrência entre os ácidos linoléico e α-linolênico, para a produção dos seus respectivos subprodutos, é determinada pela afinidade da enzima delta 6 dessaturase por ambos ácidos graxos, entretanto, ela apresenta maior especificidade pelos ácidos graxos da família ômega 3 (MADSEM *et al*., 1999). Do ponto de vista metabólico, os AGEs estão envolvidos na síntese de vários tipos de eicosanóides. A maior parte dos eicosanóides derivados da família ômega 6 pode exercer atividades pró-inflamatórias, enquanto que a maioria dos sintetizados a partir da família ômega 3 exercem funções anti-inflamatórias ou citoprotetoras (SCHMITZ & ECKER, 2008). O teor e o tipo de AGEs inseridos nos fosfolipídios de membrana dependem da quantidade e qualidade ingerida na dieta. Após a digestão e absorção entérica, os AGPIs-CL são transferidos dos enterócitos para corrente sanguínea, sendo transportados sob as seguintes formas: esterificados em triglicérides, ligados a ésteres de colesterol que compõem lipoproteínas ou ligados a proteínas como a albumina (CHEN et al., 2008; OUELLT et al., 2009).

Os principais locais extra-neuronais de síntese dos AGPI-CL em mamíferos é o fígado e, em menor escala, os astrócitos e endotélio capilar (WILLIARD et al., 2001). No feto e no neonato, o processo de alongamento e dessaturação ocorre nos astrócitos que compõem a barreira hemato-encefálica (BHE) (YEHUDA et al, 1997), apresentando maior seletividade à entrada dos AGEs (AVELLINI et al, 1994). No entanto, a quantidade de DHA sintetizado a partir do ácido α-linolênico nesse sítio é extremamente limitada, de forma que depende, em maior parte, dos estoques da mãe fornecidos pela circulação placentária e lactação (MAKRIDE et al, 2000).

Uma vez no cérebro, os AGPIs-CL são convertidos, ao nível da BHE, em acil-CoA através da enzima acil-CoA sintetase (MASHEK et al., 2006) e são preferencialmente trans-esterificados na posição sn-2 em fosfolipídios de membrana por meio da enzima acil-CoA transferase (NARIAI et al., 1994). O DHA tem sido encontrado estereficado principalmente nos fosfolipídios fosfatidiletanolamina e fosfatidilserina. Já o AA é encontrado principalmente ligado a fosfatidilinositol (CHEN et al., 2011). A liberação de AGPI-CL dos fosfolipídios de membrana é realizada pela ação de enzimas do tipo fosfolipase A2, no entanto, 90 % dos AGPI-CL podem ser re-esterificados (BAZINET et al., 2005), sofrer β oxidação mitocondrial ou ainda atuarem como biomensageiros em cascatas de sinalização para apoptose, proliferação celular e inflamação. A Figura 2 ilustra as principais etapas acima referidas.

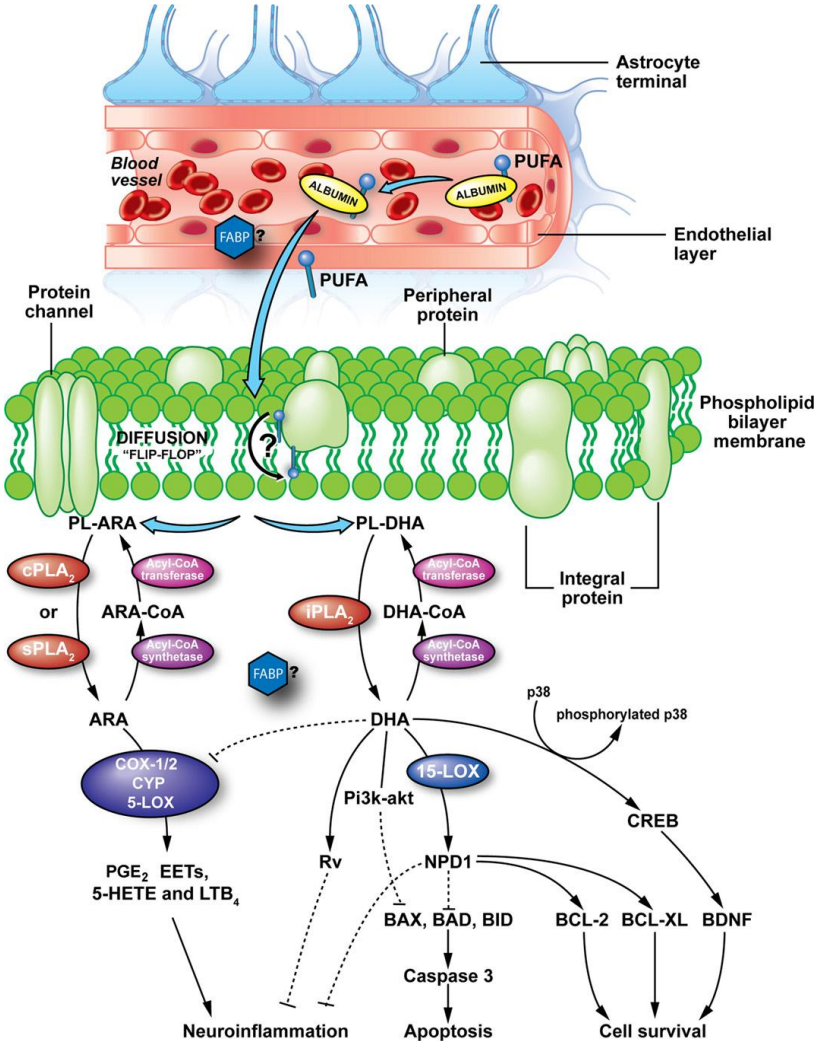


Figura 2. Mecanismos de transporte, síntese e de ação intracelular de ácidos graxos poli-insaturados. Figura retirada Bousquet et al., 2011 . BHE: barreira hematoencefálica, PUFAs: ácidos graxos poli-insaturados, FABP : transportador de ácidos graxos, PLA2: fosfolipase A2 dependente de cálcio, BDNF: fator neurotrófico derivado do cérebro, cPLA2: fosfolipase citosólica C, COX: cicloxigenase, iPLA2: fosfolipase independente de Ca2+, LOX: lipoxigenase LTB: leucotrieno B, PGE: prostaglandina E; PL: fosfolipídio; NPD1, neuroprotectina D1; Rv, resolvina; sPLA2: fosfolipase A2 secretada, BCL-2: anti-apoptótico. BAX, BAD e BID pró-apoptóticas.

O consumo de quantidades equilibradas das duas classes de AGEs é de importância, porque as mesmas podem exercer funções metabólicas opostas. Recomenda-se que o consumo dos precursores de AGEs em seres humanos, contenha uma razão ácido linoleico/ácido α-linolênico no mínimo 2:1 (SIMOPOULOS et al.,1999) e no máximo 10:1. Um aumento nesta razão pode elevar a síntese e atividade biológica de moléculas com atividade pró-inflamatória como algumas prostaglandinas, tromboxanos, leucotrienos derivados da série ômega 6 e reduzir a expressão de moléculas neuroprotetoras, os docosanóides, como resolvina e neuroprotectina D1 derivadas da série ω-3 (SCHIMITZ & ECKER, 2008). Além disso, discute-se que níveis aumentados de precursores da família ω-6 podem alterar a modulação da expressão de vários genes relacionados com o metabolismo pró-oxidante

(KITAJKA et al., 2004), fatores de transcrição e atividade de proteínas ligadas a injúrias da membrana celular (SCHIMITZ & ECKER, 2008; BAZAN 2011).

Estima-se que a razão ω 6 / ω 3 na dieta das pessoas que viveram no período que antecedeu a industrialização era em torno de 1:1 a 2:1, devido ao consumo abundante de vegetais e de alimentos de origem marinha, contendo ácidos graxos ω-3. Com a industrialização, ocorreu um aumento progressivo dessa razão, devido, principalmente, à produção de óleos refinados oriundos de espécies oleaginosas com alto teor de ácido linoleico e à diminuição de vegetais e sementes ricos em ω-3. As fontes de AGES de origem vegetal são os óleos vegetais, sendo que a maior concentração do ácido alfa-linolênico ocorre nos óleos de linhaça, de canola, soja e mustarda. O ácido α-linolênico e os AGPI-CL estão presentes em alimentos de origem animal também, principalmente nos peixes e aves, sendo as suas quantidades dependentes da dieta que esses animais foram submetidos (SIMOPOULOS, 2004). Assim, inúmeros estudos têm sido conduzidos com o objetivo de estabelecer as quantidades mais apropriadas para a incorporação do ácido α -linolênico nas rações dos animais, que possibilitem o aumento da sua conversão enzimática para AGPI-CL, resultando em maiores quantidades de EPA e DHA nestes alimentos

Entretanto, existem fontes pobres em AGEs como o óleo de coco, entre outros. A palmeira do coco babaçu é classificada genericamente como *Orbignya oleifera* por alguns botânicos, enquanto outros estudiosos a classificam como *O. Martiana*. O vegetal pertence à família Palmaceae, sub-família Ceroxylinaceae cujo nome científico é *Orbignya martiana* (ROSA, 1986). O coco de babaçu é um fruto de origem brasileira, uma planta típica da região de cerrado e do Norte-nordeste. O mesmo possui em sua amêndoa 60% de óleo rico em ácido graxo saturado láurico com concentração acima de 40%,. como ilustra a Figura 2. Esse óleo é muito empregado no preparo de gorduras especiais para confeitaria, sorvetes, margarinas e substitutos de manteiga de cacau (GETÚLIO, 2006).

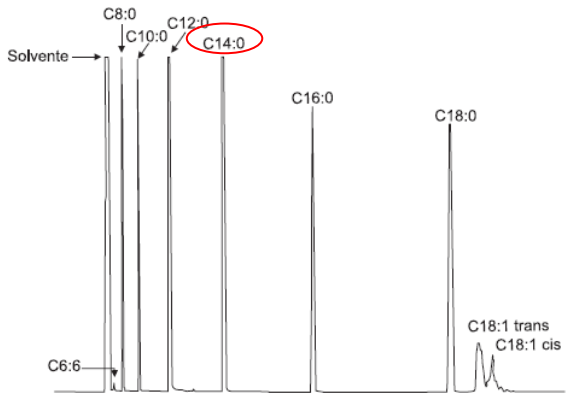


Figura 3. Cromatograma dos ácidos graxos constituintes do óleo de coco babaçu , figura retirada de GETÚLIO, 2006.. Óleo rico em ácidos graxos saturados principalmente o ácido láurico com concentração acima de 40%.

2.2. Deficiência em AGEs: um grave problema de saúde pública

A deficiência em AGEs é considerada um grave problema de saúde pública em regiões onde a desnutrição protéico-calórica (DPC) ocorre de forma acentuada. Uma análise detalhada dos efeitos deletérios provocados por estes dois tipos de má-nutrição evidenciam um ciclo-vicioso entre ambos. A DPC ou PEM (malnutrition protein energy) causa a deficiência em AGES devido ao reduzido suprimento destes ácidos graxos pela baixa ingesta, digestão, absorção e transporte dos mesmos; associado a um menor grau de dessaturação e alto metabolismo ocasionado pela β-oxidação e peroxidação. A deficiência em AGES diminui a absorção e transporte dos próprios ácidos graxos, o que negativamente afeta a DPC por comprometer a absorção de nutrientes e utilização de calorias da dieta, fechando o ciclo. A figura 4, retirada de SMIT et al., 2004, ilustra o ciclo vicioso acima descrito.

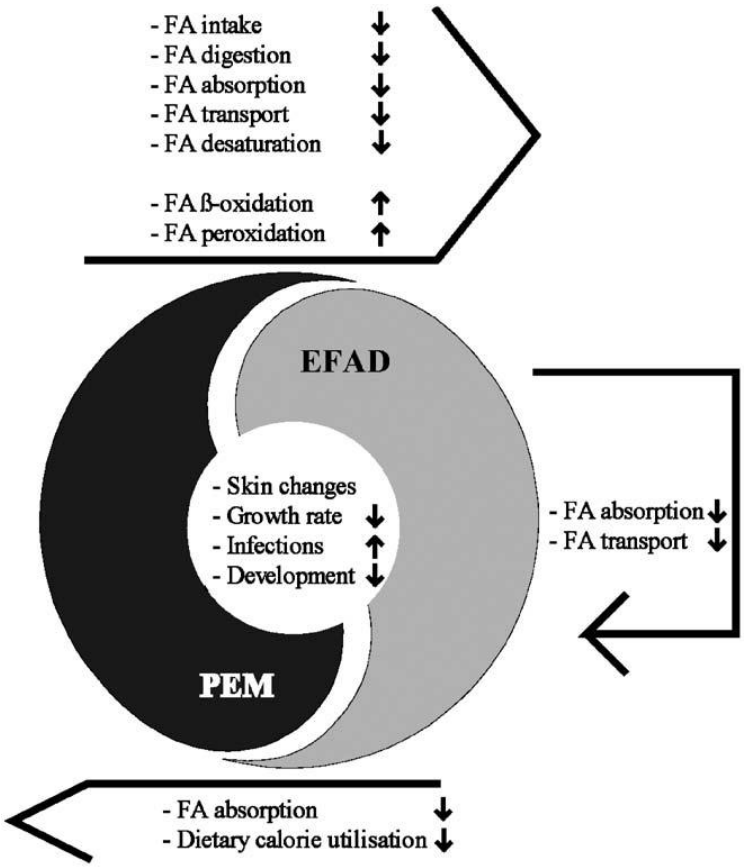


Figura 4: Ciclo vicioso entre a desnutrição energético-proteica e a deficiência em ácidos graxos essenciais. Figura retirada de SMIT et al., 2004. EFAD= Deficiência em ácidos graxos essenciais; PEM= má-nutrição energético-protéica.

Uma deficiência em AGPI-CL pode também ser gerada em síndromes metabólicas tais como Diabetes *Melitus*, intolerância a glicose, elevados índices de massa corporal em obesidade, ou ainda pela adoção de uma dieta materna muito rica em carboidratos e calorias (MUSKIET et al., 2006). Nestes casos, fluxo transplacentário aumentado de glicose pode aumentar a síntese de novo de ácidos graxos saturados tais como os ácidos palmítico e palmitoleico e monoinsaturados como o ácido oleico no feto. O ácido oléico aumentado compete com o ácido linoléico pela delta-6-dessaturase, diminuindo assim a síntese de AA, importante para o desenvolvimento encefálico. Tais alterações geram, portanto uma deficiência relativa de AGPI-CL. A figura 5 retirada de MUSKIET et al., 2006 ilustra os efeitos acima descritos.

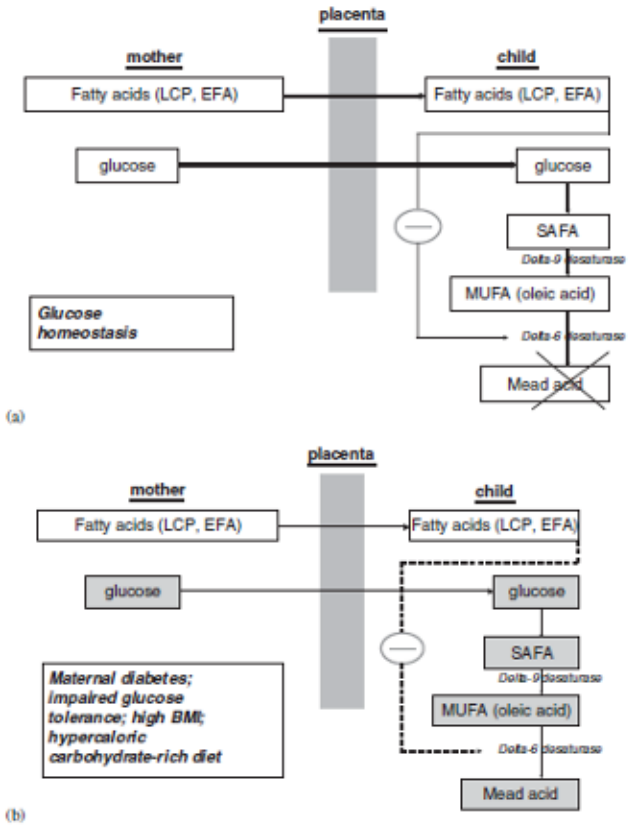


Figura 5: Status de AGE e AGPI-CL em condições normais (a) ou aumentadas de transporte de glicose transplacentária sobre a síntese de ácidos graxos da série ômega 9 (mead acid) em detrimento dos AGPI-CL (b). Figura retirada de MUSKIET et al., 2006. EFA: ácidos graxos essenciais, SAFA: ácidos graxos saturados; LCP: ácidos graxos poli-insaturados de cadeia longa, MUFA: ácidos graxoss monoinsaturado, Mead acid: 20-3n9.

2.3 A importância dos Ácidos Graxos Poli-insaturados para Sistema Nervoso Central

Depois do tecido adiposo, o sistema nervoso central (SNC) de mamíferos possui a maior concentração de lipídios do organismo, os quais correspondem cerca de 60% do peso seco do cérebro. Destes, 40% são de AGPI-CL, sendo cerca de 10-15% de AA e 10-

20% de DHA. Essas variações de percentual de AA e DHA dependem da região cerebral e da fase de desenvolvimento (VALENZUELA & NIETO, 2003).

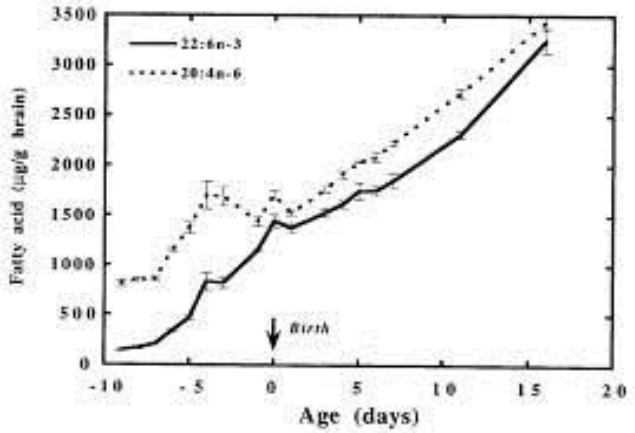
Sabe-se que durante a gestação, tanto os estoques quanto a ingesta dietética materna de ácidos graxos ômega-3 são de importância fundamental para assegurar ao neonato o fornecimento adequado destes ácidos graxos durante todo o período crítico de desenvolvimento cerebral. Um dos mecanismos de oferta de DHA ao feto, faz-se através da mobilização dos triglicerídeos armazenados no tecido adiposo materno, contendo DHA, que pode ser liberado e direcionado ao feto através do transporte placentário (HORNSTRA et al, 2000). Assim, a placenta extrai AA e DHA seletivamente e substancialmente do sangue materno e os transfere para circulação fetal (BOOTH, 1981). Durante a lactação, os AGPI-CL originam-se da ingestão dietética da mãe, dos seus estoques lipídicos e da síntese endógena a partir de seus precursores no fígado e glândula mamária (KOLETZKO, 2001). Os AGPI-CL de origem dietética que são absorvidos e reesterificados em triacilgliceróis, entram na circulação na forma de quilomícrons, sendo rapidamente transferidos para a glândula mamária pela ação da lipase lipoproteica e em seguida transferidos para o leite materno. Os triacilgliceróis hepáticos também são transportados (como VLDL) do fígado para a glândula mamária e liberados deste tecido por ação também da lipase lipoproteica. Durante o aleitamento, a atividade dessa enzima diminui no tecido adiposo e aumenta no tecido mamário, indicando um aumento da captação de ácidos graxos para este tecido (KOLETZKO, 2001).

Uma questão crucial que vem sendo apontada pela comunidade científica em muitos países, incluindo o Brasil, é a substituição do aleitamento materno pela oferta de leite bovino diluído em água à criança. O leite bovino modificado apresenta 2% de energia na forma de ácidos graxos ω-6 e 1% na forma de ácidos graxos ω-3; além disso, somente os ácidos graxos α-linolênico e linoléico estão presentes, observando-se total ausência de DHA (CONNOR, 2000). Com base nessa situação, a maior parte dos estudos atuais vem sendo dirigidos no sentido de comprovar os benefícios da utilização da suplementação de ácidos graxos poli-insaturados (PUFA) ω-3 e/ou ω-6 em formulações infantis, além de suplementar a dieta materna com estes ácidos graxos, principalmente em casos de pequeno intervalo entre estados sucessivos de gravidez.

Em humanos, o rápido depósito de DHA nos fosfolipídios de membrana ocorre durante o terceiro trimestre de vida intrauterina (CLANDININ et al, 1980) e em ratos acumulação de DHA no sistema nervoso tem início a partir do 13º dia embrionário, estendendo-se ao longo do período de aleitamento. Os períodos de maior incorporação de AGEs no sistema nervoso coincidem com os estágios de intensa neurogênese, gliogênese, migração neuronal, diferenciação, sinaptogênese e mielinização (GREEN et al., 1996). Sob

condições de deficiência dietética materna de α-linolênico, há uma substituição compensatória do DHA pelo subproduto da serie ômega 6, o ácido docosapentaenóico (DPA) no tecido nervoso do concepto (GALLI et al., 1971). Isso resulta na alteração da relação ω-6/ω-3 e consequentemente na modificação da composição de AG nos fosfolipídios de membranas neuronais e células da glia (BOURRE et al., 1990). A figura 6A ilustra a agregação de DHA e AA no encéfalo ao longo do desenvolvimento embrionário e pós-natal de ratos (GREEN et al., 1996). A figura 6B ilustra como a similar incorporação de DHA no córtex cerebral de humanos está relacionada aos períodos de desenvolvimento encefálico, principalmente com a expansão inicial de desenvolvimento da substância cinzenta que continua até 12 anos e depois sofre um declínio ao longo da vida (MAcNAMARA & CARLSON, 2006).

A



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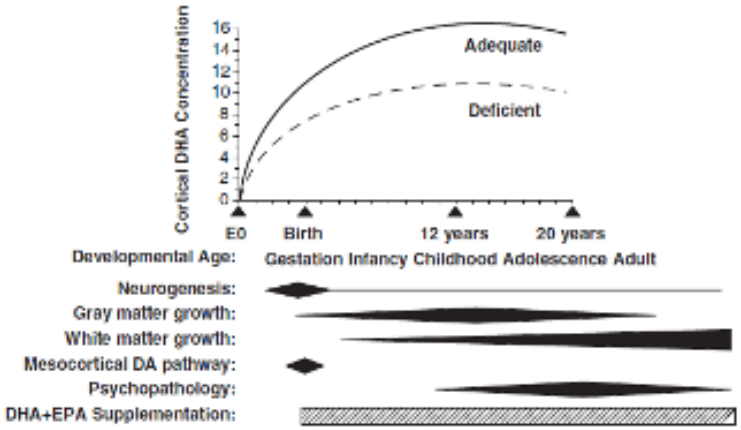


Figura 6. (A) Incorporação de ácidos graxos poli-insaturados (DHA e AA) durante o desenvolvimento embrionário e pós-natal do encéfalo de ratos. (B) Concentração de DHA no córtex de humanos do período embrionário até 20 anos. Fig. A retirada de GREEN et al., 1996 e Fig B retirada de MAcNAMARA E CARLSON, 2006.

Os AGPI-CL desempenham importantes funções estruturais e funcionais não só durante o desenvolvimento, mas também durante a vida adulta e envelhecimento do sistema nervoso, devido aos seus efeitos neuroprotetores (KALMIJN et al., 1997; DYALL et al., 2007; ZHE YING et al, 2012). Um dos mecanismos envolvidos com a neuroproteção no adulto está relacionado com a ativação, através do DHA, de fatores de transcrição pela ação do receptor retinóide X (RXR) que está envolvido com a neurogênese tardia que ocorre no hipocampo de adultos (CHAMBRIER et al., 2002). Evidências apontam que os RXR controlam a função de células responsáveis pela inflamação no SNC, a microglia, como também inibem a produção de óxido nítrico (DHEEN et al., 2005) protegendo neurônios da morte celular.

2.4 Sistema Dopaminérgico e Deficiência Dietética em Ácidos Graxos Essenciais

Os AGPI-CL exercem atividades modulatórias sobre vários sistemas de neurotransmissores, incluindo o glutamatérgico (MILLER et al., 1994), GABAérgico (HAMANO et al., 2006) e o sistema monoaminérgico (CHALON et al., 2006; VINES et al., 2010). Dentre estes sistemas, o dopaminérgico é bastante sensível à deficiência específica de AGEs (CHALON, 2006).

A deficiência crônica de DHA por três gerações reduziu a liberação vesicular de dopamina (DA) no núcleo acumbens e córtex frontal de ratos onde foi também observada redução na densidade de vesículas sinápticas contendo DA (ZIMMER et al., 2000). Similar deficiência em DHA foi capaz de reduzir a densidade de receptores dopaminégicos do tipo D2 no córtex frontal de ratos adultos e jovens (DELION et al., 1994). Evidência mais recente, no entanto, demonstrou que este tipo de insulto nutricional aumenta a densidade de receptores D2 na substantia nigra, corpo estriado, amígdala, hipocampo (YAVIN, 2009)

Animais submetidos a uma restrição dietética moderada em AGEs exibiram alterações expressivas em padrões comportamentais, indicativas de disfunção dopaminérgica, sendo algumas destas alterações revertidas pela suplementação da dieta após o período de desmame (LEVANT et al., 2004). Diferentes tipos de alterações neuroquímicas e comportamentais relacionadas a mudanças no sistema dopaminérgico mesolímbico e meso-estriatal foram também observadas por níveis mais severos de deficiência em DHA (FEDOROVA et al., 2006; KUPERSTEIN et al., 2008; LEVANT et al., 2010). Um modelo que associa gestações sucessivas a uma dieta com reduzidos níveis de ácido α-linolênico foi

capaz de reduzir o número de células dopaminérgicas da substância negra compacta e área tegmentar ventral de animais adultos (AHMAD et al., 2008).

Recentemente, em nosso laboratório de Neurofisiologia, evidências demonstraram em animais jovens, uma vulnerabilidade diferenciada em duas populações de neurônios dopaminérgicos da *substantia nigra* à restrição dietética em AGES por duas gerações (PASSOS et al., 2012).

2.5 Importância do sistema dopaminérgico nos circuitos que envolvem os núcleos da base

Os Núcleos da Base (NB) são um termo coletivo utilizado para descrever um grupo de núcleos subcorticais interconectados que recebem e fornecem alças de feedback para várias áreas do córtex cerebral e também influenciam alças descendentes a regiões motoras do tronco cerebral. A ação exercida pelos neurônios dopaminérgicos nos circuitos dos NB permite que a mesma module a atividade de várias alças de retroalimentação com o córtex (BEAUREGARD & FERRON, 1991), favorecendo atividades límbicas, cognitivas, oculomotoras e de consolidação de memória ilustrados na Figura 7.

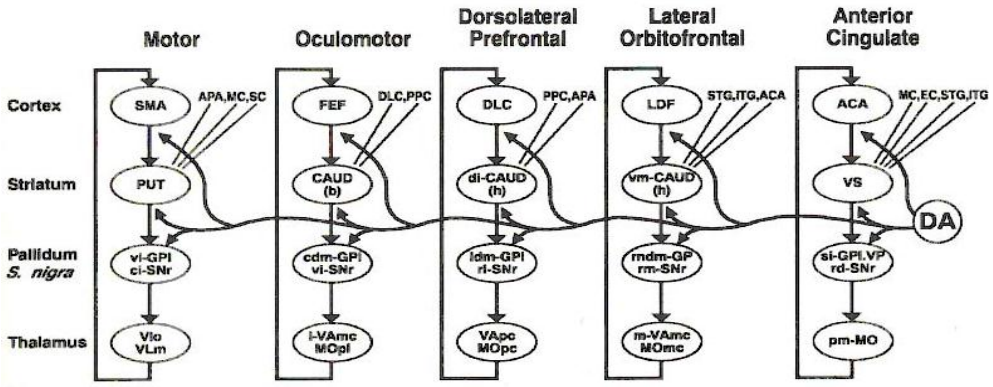


Figura 7. Alças relacionadas com funções motoras, límbicas e cognitivas envolvendo os núcleos da base e diferentes regiões corticais, onde a dopamina (DA) exerce atividade modulatória. Alças de retroalimentação dos Núcleos da Base com diferentes regiões do córtex cerebral envolvidas no controle motor, oculomotor, cognitivo (dorsolateral prefrontal e lateral orbitofrontal) e límbico (anterior cingulate). A DA mesencefálica exerce uma ação modulatória sobre todas essas alças. Figura retirada de BEAUREGARD & FERRON, 1991.

O principal aferente excitatório aos NB é o córtex motor. O córtex envia projeções excitatórias ao estriado, o principal núcleo de entrada dos NB. Fibras córtico-estriatais fazem sinapses com neurônios GABAérgicos de projeção, chamados neurônios médios espinhosos. As projeções destes neurônios constituem duas alças funcionais. Uma parte deles projeta-se ao Globo pálido interno (GPi) e à substância negra pars reticulata (SNr) (DELONG et al 2007). Esta é a via direta que fornece um controle inibitório direto sobre os

núcleos de saída dos NB. O controle inibitório sobre as células GABAégicas de saída do GPi/SNr fornecido pela via direta leva a uma desinibição das projeções tálamo-corticais.

A outra parte dos neurônios médios espinhosos estriatais participa de uma projeção multissináptica conhecida como via indireta. Estes neurônios que originam a via indireta projetam-se para o globo pálido externo (GPe) e inibem as células GABAérgicas deste núcleo. Estas células normalmente exercem um controle inibitório tônico sobre as células glutamatérgicas do núcleo subtalâmico (NST), mas a ativação da via GABAérgica estriato-palidal leva a uma desinibição do NST. Isso permite uma transmissão excitatória entre o NST e os núcleos de saída dos NB, o GPi/SNr, desta maneira inibindo as projeções tálamo-corticais (BERGMAN et al., 1990). As vias direta e indireta dos NB atuam como um mecanismo de fino ajuste no controle do movimento. O balanço da transmissão através destas vias é fortemente regulado por uma projeção modulatória a partir de neurônios dopaminérgicos da substância negra parte compacta (SNc). Esta entrada dopaminérgica ao estriado regula diferentemente as vias direta e indireta devido à presença de diferentes receptores dopaminérgicos pós-sinápticos (BERGMAN et al., 1990). Existem cinco tipos de receptores de DA, que podem ser subdivididos em receptores D1 (subtipos D1 e D5) localizado na via direta e em D2 (subtipos D2, D3 e D4) na via indireta .

A dopamina também modula a ativação de interneurônios nitrérgicos do estriado. Vários estudos têm indicado que interneurônios produtores de óxido nítrico (NO) desempenham um importante papel na regulação da transmissão sináptica córtico-estriatal e comportamento motor. A síntese de NO é estimulada pela ativação paralela de receptores D1 e NMDA sendo capaz de aumentar a excitabilidade neuronal e facilitar a transmissão glutamatérgica cortico-estriatal. Paralelamente, a sinalização do NO se opõe aos efeitos inibitórios da ativação do receptor D2 na via indireta (WEST & TSENG, 2011). A figura 8 esquematiza a participação dos receptores D1 e D2 e dos interneurônios nitrérgicos nas vias diretas e indiretas dos circuito dos NB.

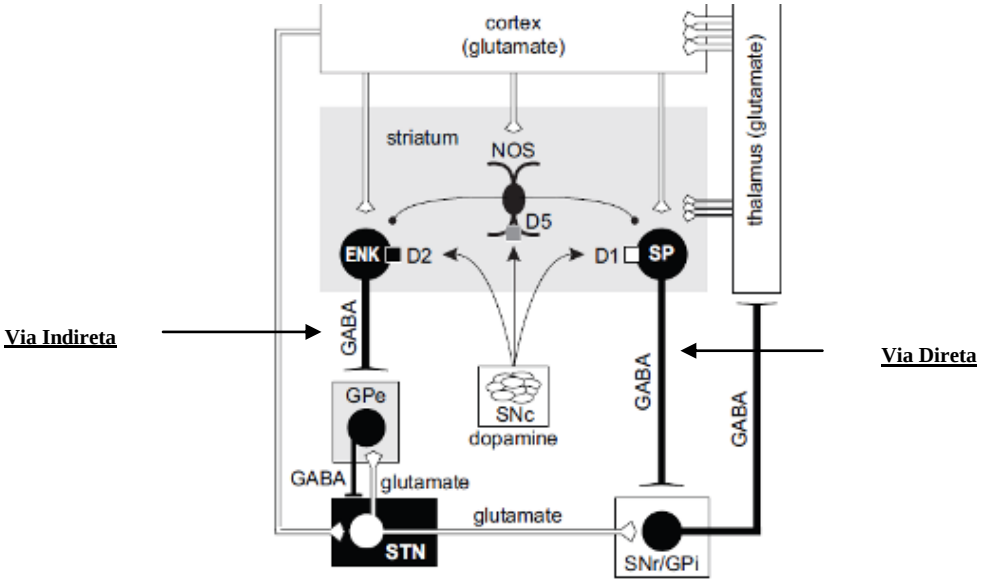


Figura 8: Diagrama ilustrando as principais conexões dos Núcleos da Base envolvidas com a ativação dos receptores D1 na via direta e D2 na via indireta. Interneurônios contendo óxido nítrico são capazes de modular a atividade destas vias . Figura retirada de WEST & TSENG, 2011.

2.6 Mecanismos Envolvidos na Neurodegeneração das Células Dopaminérgicas na Substância Negra

O processo de neurodegeneração envolve múltiplos fatores que de forma conjunta podem levar à morte celular. Um mecanismo bastante estudado atualmente é o envolvimento da disfunção mitocondrial na perda de neurônios dopaminérgicos.

As mitocôndrias são organelas onde ocorrem o metabolismo energético oxidativo, a homeostase do cálcio e o controle da morte celular programada, apoptose (GALLUZZI et al, 2009). A disfunção mitocondrial tem sido amplamente relacionada com a perda de neurônios dopaminérgicos envolvida na patogênese da Doença de Parkinson (DP) (ABOU-SLEIMAN et al., 2006). A deficiência energética relacionada à mitocôndria pode levar ao rompimento de vesículas que armazenam DA, aumentando sua concentração no citosol, ocasionando danos em macromoléculas (DAUER & PRZEDBORSKI, 2003).

Apoptose dos neurônios dopaminérgicos nigrais é um evento envolvido no processo de neurodegeneração da PD (BATTIST et al., 2008). O processo apoptótico é causado por uma cascata de eventos na qual a família de cisteína proteases conhecida como caspases (cysteinyl aspartate-specific proteinase) levam a quebra de múltiplos substratos, com especificidade para o ácido aspártico (LEV et al., 2003). Achados clínicos, estudos com modelos experimentais da DP, bem como estudos *in vitro* de várias linhagens celulares demonstram o envolvimento da apoptose na neurodegeneração dos neurônios dopaminérgicos (SINGH & DIKSHIT, 2007). Existem duas vias que relacionam a contribuição mitocondrial com a morte celular. A primeira via envolve a permeabilização da membrana mitocondrial externa (MOMP) e consequente liberação do citocromo c (cyto c); isso ocorre quando são ativadas as proteínas pró-apoptóticas da família Bax e/ou Bak (GREEN, 2005). A segunda via de morte celular é iniciada por condições que levam à transição de permeabilidade mitocondrial (MPT), definido como um aumento não específico da permeabilidade da membrana mitocondrial interna (HALESTRAP, 2000), levando ao inchaço da organela e ruptura da membrana externa (GREEN, 2005).

Um mecanismo clássico importante no processo neurodegenerativo, o estresse oxidativo (EO), é descrito como o desequilíbrio entre a formação e a eliminação de espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) (BARNHAM et al., 2004). As ERO (O_2^-, ânion superóxido; OH^-, radical hidroxil; H_2O_2, peróxido de hidrogênio) e as RNS ($ONOO^-$, peroxinitrito e NO, óxido nítrico) são agentes responsáveis pelas principais causas do estresse oxidativo no SNC (ROSA, RM et al, 2004). Aproximadamente 100% do oxigênio molecular são consumidos pela respiração

mitocondrial formando como subprodutos espécies oxidantes incluindo ERO (DAUER & PRZEDBOSKI, 2003). Dentre os tipos de EO, existe o processo de lipoperoxidação que pode ser desencadeado pelo ataque dos radicais livres aos lipídios poli-insaturados via enzimática (ciclooxigenases e peroxidases) ou não-enzimática (auto-oxidação) , levando a produção de subprodutos a exemplo do malodialdeído, um produto secundário da via enzimática, derivado da β-ruptura dos ácidos graxos poli-insaturados (DEL RIO et al., 2005). Por outro lado, níveis equilibrados de radicais livres no organismo, apresentam importantes papéis a exemplo das ações bactericidas, fungicidas e antivirais, promovendo, assim, uma barreira de defesa frente à presença de microorganismos patogênicos (OKTYABRSKY et al., 2007). Ademais, recentes descobertas demonstram que os radicais livres podem atuar como segundos mensageiros das vias específicas na sinalização celular em vários processos fisiológicos como a transcrição gênica (DEMPLE et al., 1999).

Outro fator de grande relevância é a homeostase das proteínas que estão relacionadas aos mecanismos neurodegenerativos, pois a qualidade e quantidade, como o balanço na síntese, maturação e degradação dessas macromoléculas mantêm o equilíbrio metabólico cerebral (SHAH & Di NAPOLI, 2007). Por exemplo, a α-sinucleína, uma proteína pré-sináptica de importância na DP, exerce toxicidade quando encontra-se sob a forma de fibrilas agregadas (corpos de Lewy) (FREDENBURG et al., 2007).

Outro ponto bastante discutido acerca dos fatores envolvidos com a lesão celular é a neuroinflamação. Estudos realizados utilizando SN de pacientes com doença de Parkinson evidenciaram um aumento da reatividade das células microgliais nesta região (McGEER et al., 1988). A microglia, célula de defesa imune no SNC, quando ativada produz citocinas tais como o fator de necrose tumoral (TNF) α, interleucina (IL)-1β e interferon γ (BOKA et al.,1994), as quais podem induzir o aumento na atividade das enzimas óxido nítrico sintase, ciclo-oxigenase 2 e NADPH-oxidase, de forma a produzir altas quantidades de ERO (HUNOT et al., 1996;. KNOTT et al, 2000.).

A excitotoxidade também faz parte do cenário de indução de morte celular. Dados experimentais indicam que o glutamato pode contribuir para degeneração de células por meio da disfunção dos astrócitos (DERVAN et al., 2004). Estas células da glia são imprescindíveis para manter os níveis adequados de glutamato extracelular através da captação do mesmo via transportadores, auxiliando no metabolismo e reciclagem do excesso de glutamato, através da atividade da enzima glutamina sintetase. Com isso, há preservação dos níveis ideais de glutamato extracelular, prevenindo a indução de morte celular por aumento do influxo de cálcio e consequentemente ativação das caspases (PLAITAKIS & SHASHIDHARAN, 2000; DERVAN et al, 2004).

2.7 Óxido Nítrico e Neurodegeneração

Alguns estudos têm demonstrado o envolvimento do NO na degeneração de neurônios dopaminérgicos da via nigroestriatal (DUNCAN & HEALES, 2005). O NO é um neurotransmissor que em certas condições pode atuar como radical livre de vida curta (1-5 segundos), altamente permeável às membranas biológicas por ser uma molécula gasosa, tendo sido implicado em várias condições fisiológicas (processos de sinalização e comunicação celular em uma variedade de sistemas) e patológicas, incluindo a morte celular (IRAVANI et al., 2002; CALABRESE et al., 2007). O NO é sintetizado a partir do aminoácido L-arginina pela enzima NOS que apresenta três formas distintas (isoenzimas): a forma neuronal (NOSn; tipo I), a forma induzida (NOSi; tipo II), que é produzida em grande quantidade pela microglia e astrócitos reativos além de macrófagos e a forma endotelial (NOSe; tipo III). O encéfalo expressa as três isoformas da enzima NOS, e embora cada isoforma possa desempenhar um discreto papel fisiológico, o NO gerado pode atuar tanto em processos fisiológicos como patológicos no SNC (DUNCAN & HEALES, 2005). Os níveis de NOSi no SNC são baixos, mas essa isoforma pode ser induzida em algumas condições patológicas que envolvem estresse oxidativo (CALABRESE et al., 2007). Há evidências de que o NO em excesso pode está associado com o processo de excitotoxicidade, danos ao DNA e modificações de proteínas, os quais podem promover mecanismos patogênicos em processos neurodegenerativos (ZHANG et al., 2005).

A NOSn e a NOSe são enzimas constitutivas e dependentes de cálcio/calmodulina, enquanto a expressão da NOSi é cálcio/calmodulina independente e requer estimulação apropriada como citocinas (HENEKA et al., 2000). A ativação da NOSn e da NOSe produz níveis relativamente baixos de NO por curtos períodos de tempo, enquanto a indução da NOSi catalisa altos níveis de NO por longos períodos de tempo. No SNC, a NOSn é responsável pela maior produção de NO em condições fisiológicas (CALABRESE et al., 2007).

O NO pode exercer função dual no SNC: a baixos níveis o NO exerce função como neurotransmissor e atua na diferenciação celular durante processos fisiológicos (BRENMAN & BREDT, 1996; FEELISCH et al., 1994; HOBBS & IGNARRO, 1996), entretanto, o NO pode atingir concentrações entre 50nM a 500nM (PACHER et al., 2007), podendo causar efeitos deletérios através da citotoxidade. Durante estágios patológicos, a ativação da microglia e astrócitos é desencadeada pela ativação da

NOSi , liberando elevadas concentrações de NO ~1µM (HALL et al, 1998). Os radicais O_2^- e OH^- podem interagir com o NO formando $ONOO^-$, estes são responsáveis por promover danos celulares ao reagirem com ácidos nucléicos, proteínas e lipídios (DAUER & PRZEDBORSKI, 2003).

O estudo do efeito protetor ou lesivo do NO sobre neurônios tem sido amplamente debatido (CALABRESE et al., 2007). Em diversas doenças neurodegenerativas como a esclerose lateral amiotrófica e a DP, o NO em altas concentrações leva à morte celular por apoptose de neurônios dopaminérgicos (SINGH & DIKSHIT, 2007). Por outro lado, concentrações fisiológicas do NO suprimem eventos apoptóticos como aqueles induzidos por hipóxia (SINGH & DIKSHIT, 2007). Entre os efeitos citotóxicos do NO e/ou do $ONOO^-$ estão os danos ao DNA, acúmulo do supressor tumoral e fator de transcrição nuclear p53, além da indução da liberação do citoromo c (cyto c), ativação de caspases e modulação do efeito da ciclooxigenase-2 (COX-2) no SNC (SINGH & DIKSHIT, 2007; CALABRESE et al., 2007).

Na DP e em outras doenças neurodegenerativas, existem evidências de que o alvo do NO e de seus metabólitos seja a mitocôndria, onde essas espécies levam à inibição da cadeia respiratória. De acordo com GIULIVI e colaboradores (2006), o NO é uma molécula sinalizadora que regula o consumo de oxigênio pela mitocôndria bem como a produção de EROS.

2.8 A influência do DHA sobre a glia em processos neurodegenerativos

As células da glia são o maior constituinte do cérebro adulto em mamíferos. A glia são responsáveis pela manutenção de um microambiente ideal para os neurônios, pois mantém a concentração externa de potássio (BENVENISTE et al., 1992), possui um grande estoque de glicogênio (CATALDO et al., 1986), oferece suporte de defesa por produção de citocinas e participa da formação da barreira hemato-encefálica (JANZER, 1987). Fatores solúveis como neurotrofinas, glicose e DHA podem também serem secretados e direcionados para os neurônios. Muitos eventos importantes como neurogênese, mielinização, sinaptogênese e diferenciação neuronal são também coordenados pela glia (GARCIA-ABREU, 1995; PARPURA et al., 2012).

Como relatado anteriormente, um dos tipos de célula da glia, o astrócito, desempenha papel crucial na proteção dos neurônios contra a excitotoxicidade causada por excesso de glutamato e também de amônia (PLAITAKIS & SHASHIDHARAN, 2000), além de realizar a síntese de glutationa e de enzimas antioxidantes (AOYAMA et al., 2008). Por outro lado,

sabe-se que essas células neuronais possuem um papel crucial nos processos de dano cerebral. Eles podem responder aos processos patológicos, juntamente com a microglia, através de um processo chamado gliose ou astrogliose. A *substantia nigra* exibe grandes quantidades de microglia em condições normais sendo estas bastante reativas em cérebros de humanos com a Doença de Parkinson (McGEER et al., 1988; BLANDINI, 2013). A microglia ativada produz não apenas citocinas, mas também altos níveis de óxido nítrico e radicais superóxidos (MINGHETTI et al., 1999). Esses fatores têm sido apontados como agentes potencializadores no processo de neurodegeneração (McGEER et al., 1988; DEXTER , 2013). Um estudo recente demonstrou que a deficiência nutricional em ácido alfa-linolênico é capaz de induzir proliferação de células da microglia em regiões como hipocampo, córtex frontal, amígdala e corpo estriado em animais adultos jovens (KURPERSTEIN et al., 2008). Por outro lado, este tipo de dano aumenta a astrogliose que normalmente ocorre no hipocampo em animais idosos (LAVIALLE et al., 2013). Assim, a disfunção das células da glia, induzidas pela deficiência em AGEs da família ω-3, pode aumentar a susceptibilidade do cérebro ao estresse oxidativo e neurodegeneração.

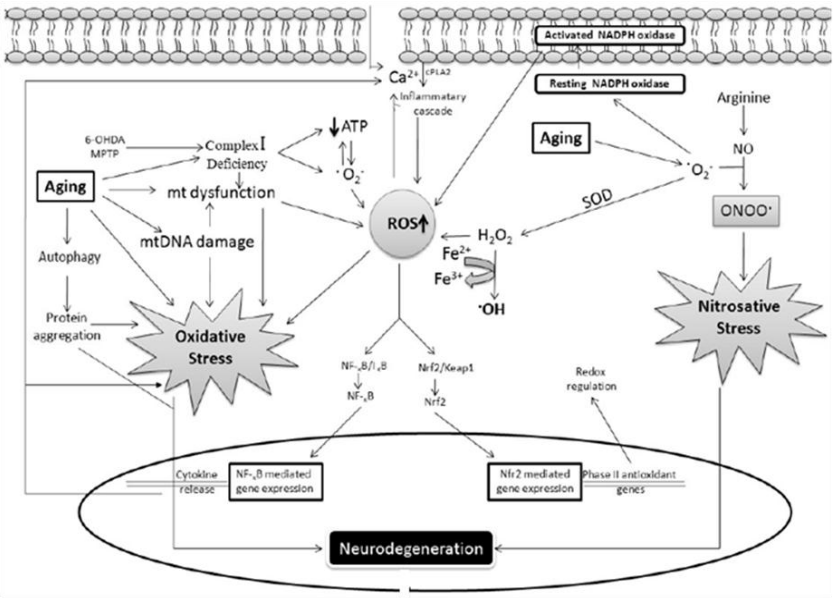
2.9 Sistemas de defesas antioxidantes

Para evitar a formação de ERO, assim como reparar os danos oxidativos em tecidos e macromoléculas, o organismo possue um complexo sistema de defesa antioxidante. Algumas destas defesas são enzimas: superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), mas existem também as defesas antioxidantes não enzimáticas, como glutationa e as vitaminas A, C e E. A enzima SOD converte o ânion $O_2^{\cdot}$ em H_2O_2 (MOLINA et al., 2003). Todos os subtipos de SOD apresentam, pelo menos, um metal de transição em seu sítio ativo como zinco e manganês. A manganês-SOD está localizada na membrana mitocondrial interna, já a cobre, zinco-SOD apresenta uma localização citosólica. As expressões dos dois tipos de SOD, ou seja, os níveis de SOD-total são regulados pela produção de ERO (HALLIWELL & GUTTERIDGE, 1999).

A CAT, uma enzima que possui o grupo heme em sua molécula, tem o H_2O_2 como único substrato, sendo sua atividade também intimamente relacionada com a concentração das ERO. A catalase tem como função principal hidrolisar o H_2O_2, formando H_2O e oxigênio molecular. Esta enzima atua de forma compelmentar à GPx, não permitindo a produção de radical hidroxil a partir do H_2O_2, que ocorre na presença de Fe^{+2} (Reação de Fenton). A GPx, uma enzima selênio-dependente, é importante para a proteção contra peróxidos orgânicos e H_2O_2. Para a sua atividade, a GPx, necessita da presença de glutationa reduzida como um importante cofator (HALLIWELL & GUTTERIDGE, 1999), sendo os níveis

homeostáticos de glutationa necessários para a sobrevida celular. Estudos em ratos e humanos têm indicado uma progressiva diminuição da função antioxidante incluindo a SOD e CAT na *substantia nigra* durante o envelhecimento do cérebro (KOSOLOVA et al., 2003; VENKATESHAPPA et al., 2012). A Figura 9 A mostra os mecanismos de formação de espécies reativas de oxigênio e 9 B a atividade das enzimas antioxidantes.

A



B

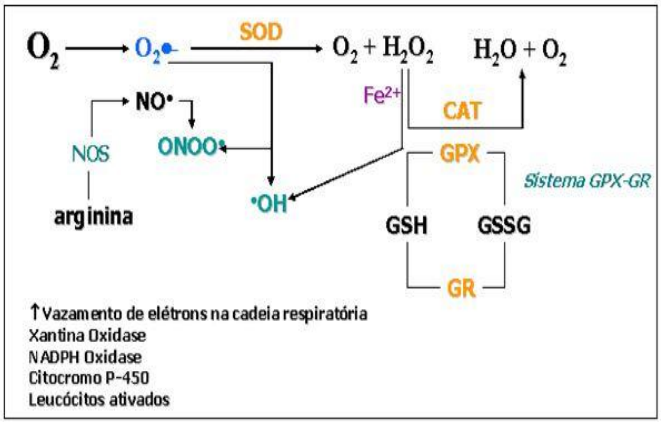


Figura 9. Mecanismos de formação de espécies reativas de oxigênio (A) (Hemant Kumar et al.,2012). Nota-se que o aumento de consumo de oxigênio possibilita vazamento de elétrons na cadeia respiratória mitocondrial, o que potencializa a geração das espécies reativas. Enzimas como xantina oxidase, NADPH oxidase, bem como os eventos decorrentes de processos inflamatórios , contribuem para geração elevada dos radicais livres. As estratégias enzimáticas antioxidantes (B) (ANTUNES NETO, 2005).

Além disto, estudos recentes também mostram uma redução progressiva ao longo da vida, nos níveis de enzimas antioxidantes na substancia negra, o que a torna especialmente vulnerável a lesões (MITHRI et al., 2011). Níveis reduzidos de glutationa (GSH), principal sistema redox de controle do estresse oxidativo, foram também observadas em amostras *post mortem* na SN de pacientes parkinsonianos (EMERIT et al., 2003).

2.10. Importância dos derivados do DHA e da neurotrofina BDNF para a sobrevida celular

O DHA pode ser oxidado e metabolizado através da enzima lipoxigenase-15, em subprodutos chamados docosanóides. O principal deles é a neuprotectina D1 (NPD1), a qual atua como agente neuroprotetor em condições de inflamação e neurodegeneração (MARCHESELLI et al., 2003). Sabe-se que ela atua ao nível mitocondrial prevenindo morte celular por apoptose, principalmente em células expostas ao insulto oxidativo. Em estudos experimentais observou-se que a NPD1 apresentou efeitos anti-apoptóticos em células do epitélio pigmentar da retina através da inclusão desse docosanóide na ordem de nanomolar, cujo resultado levou à redução dos danos causados pelo H_2O_2. Este efeito foi acompanhado da atenuação da ativação da caspase–3 e dos níveis dos agentes pró-apoptóticos Bad e Bax, bem como inibiu a ativação do gene para enzima COX-2, além da regulação negativa do fator inflamatório NF-kB. (MUKHERJEE et al., 2004). A Figura 10 abaixo ilustra a síntese de NPD1.

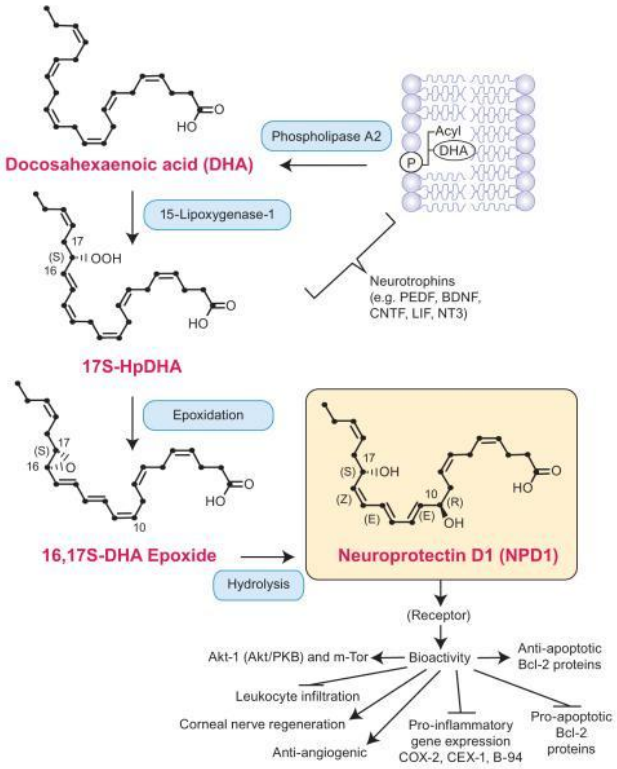


Figura 10. Mecanismo de Síntese de neuroprotectina D1. Figura retirada de BAZAN, 2011.

Evidências indicam que o DHA e possivelmente seus metabólitos, como NPD1 atuam sobre o receptor nuclear Nurr1, altamente expresso no mesencéfalo ventral. Esse receptor é vital para o desenvolvimento, migração e sobrevida dos neurônios dopaminérgicos (JANKOVIC et al. 2005). Através da heterodimerização com o fator de transcrição RXR mantém a integridade da função neuronal em adultos através da regulação dos níveis de produção de dopamina e de seus transportadores (EELLS et al. 2002; BANNON et al. 2004), bem como os genes que codificam o GDNF e BDNF (SAARMA & SARIOLA 1999; ROUSSA & KRIEGLSTEIN, 2004; BARKER et al., 2005; BARROSO-CHINEA et al., 2005).

Além da neuroprotectina D1, outros metabólitos de efeitos importantes para a proteção e plasticidade celular têm sido encontrados nas membranas de neurônios. Uma molécula recém-descoberta, a N-docosahexaenoiletanolamida (DEA) derivada do DHA estimula o crescimento de neuritos e a sinaptogênese em cultura de neurônios do hipocampo de rato. Estes efeitos celulares podem atuar nas funções de aprendizagem e memória (HEE-YONG KIM et al., 2011).

Acredita-se que níveis reduzidos de DHA e seus derivados estão associados com capacidade de recuperação das funções motoras pós-lesão medular. Um estudo realizado com modelo experimental de injúria em medula espinhal de roedores submetidos à dieta deficiente em DHA revelou uma significativa diminuição dos níveis do fator neurotrófico derivado do cérebro (BDNF) e do seu receptor no TrkB (ZHE YING et al., 2012). O BDNF, bem como outros importantes peptídeos, tais como o fator neurotrófico derivado da glia (GDNF) e o Neurturin (NTN) regulam o desenvolvimento, a viabilidade celular e exercem papel restaurador das funções neuronais (TANRIOVER et al., 2010).

Em cultura de neurônios dopaminérgicos, observou-se que o BDNF promoveu a diferenciação celular e supressão de apoptose em população neuronal na SN (BAQUET et al. 2005). O mecanismo de sobrevivência parece ser o realizado por mecanismos de sinalização através do trifosfato de inositol (IP3) e pela via AKT após ligação do BDNF com seu respectivo receptor. O DHA, fornecido ao neurônio através dos astrócitos, exerce grande contribuição na sinalização através da via da TkrB/AKT (MOHAMMED AKBAR et al., 2005). Outros estudos tem mostrado a ação do BDNF sobre a sobrevida neuronal através da ativação da sinalização PKA/CREB, o qual regula vários genes responsáveis pela neuroplasticidade (BERTON et al., 2006). Sendo assim, essa neurotrofina pode atuar sobre a neurogênese adulta e proteger os neurônios após a lesão (CRAWFORD et al., 1976). Acredita-se que as neurotrofinas atuam na manutenção do perfil fenotípico, agindo de forma sinérgica ou específica sobre os neurônios dopaminérgicos mesencefálicos (BAQUET et al.,

2005; STAHL et al., 2011). Ademais, estudos revelam que níveis reduzidos de BDNF estão associados com a fisiopatologia da ansiedade e da depressão (ALTAR, 1999; KOPONEN et al., 2005; CASTREN et al., 2007).

O tratamento em ratos com uma deficiência dietética de ácido alfa-linolênico, mesmo por um curto período de 4 semanas foi capaz de reduzir os níveis do BDNF no estriado (MIYAZAWA et al., 2010) e por um período maior, no córtex frontal (RAO et al., 2007). Menor concentração desta neurotrofina foi encontrada em cérebros *post-mortem* de pacientes com Doença de Parkinson (MOGI et al., 1999; PARAIN et al., 1999). Estudos *in vitro* utilizando culturas primárias de mesencéfalo têm indicado que a supressão de BDNF e GDNF foi capaz de induzir morte de neurônios dopaminérgicos por uma via que não envolveu a despolarização de mitocôndria e a liberação de citocromo C, mas teve a participação dos receptores de membrana envolvidos nas vias extrínsecas de apoptose. No entanto, não se sabe se o mesmo ocorre em condições *in vivo* (YU et al., 2008). Sendo assim, alteraçoes relacionadas aos níveis de fatores neurotróficos podem estar associadas a eventos como estresse oxidativo, excitotoxicidade e apoptose os quais favorecem o processo neurodegenerativo.

3. Justificativa e Hipóteses do Presente Estudo

Recentes evidências obtidas em nosso laboratório, utilizando um modelo de restrição dietética em AGEs por duas gerações revelaram uma vulnerabilidade diferenciada entre duas subpopulações de neurônios dopaminérgicos da SN a este insulto nutricional. Tal tratamento dietético reduziu em cerca de 20% o número de neurônios dopaminérgicos da região rostro-dorso-medial da SN, mas não da região caudo-ventro-lateral em animais jovens. Além disto, afetou o crescimento de todos os neurônios dopaminérgicos e reduziu os níveis proteicos da enzima Tirosina hidroxilase (PASSOS et al., 2012). Considerando que a SN apresenta propriedades bioquímicas peculiares que conferem à mesma uma alta vulnerabilidade a insultos oxidativos quando comparado com outras regiões do cérebro (KIDD, 2000), o presente estudo foi conduzido para testar a hipótese de que aumentados níveis de estresse oxidativo e reduzidos níveis de neurotrofinas podem ser potenciais mecanismos envolvidos na perda de células dopaminérgicas da SN, previamente descritas. Para testar se a depleção progressiva nos níveis de DHA, induzidos pela deficiência crônica em AGES, poderia induzir perda de células dopaminérgicas da região caudo-ventro-lateral e comprometer outros tipos de neurônios da *substantia nigra*, analisamos também os animais adultos da segunda geração.

4. OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar mecanismos relacionados com a perda de neurônios dopaminérgicos induzida pela deficiência crônica em AGEs na substância negra de ratos *Wistar* jovens e adultos.

.

4.2 OBJETIVOS ESPECÍFICOS

Investigar em ratos albinos:

a) Indicadores de neurodegeneração na *substantia nigra* e *corpus estriatum*;

b) Níveis de peroxidação lipídica na *substantia nigra* e *corpus estriatum*;

c) Atividade das enzimas antioxidantes superóxido dismutase e catalase na *substantia nigra* e *corpus estriatum*;

d) Níveis de nitrito como indicador da produção de óxido nítrico na *substantia nigra* e *corpus estriatum;*

e) Distribuição e número de células dopaminérgicas em animais adultos na *substantia nigra*;

f) Distribuição de neurônios imunorreativos ao BDNF na *substantia nigra.*

5. Artigo científico intitulado: ”Differential vulnerability of substantia nigra and corpus striatum to oxidative insult induced by reduced dietary levels of essential fatty acids”, publicado na revista *Frontiers in Human Neuroscience*, Volume 6, artigo 249, pag. 1-10; 2012.

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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, clearead 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 ± 1.87
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)	14.08 ± 0.27	13.12 ± 0.34	13.74 ± 0.83	14.93 ± 0.48
C23	3.88 ± 0.13	3.46 ± 0.18	3.98 ± 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.*

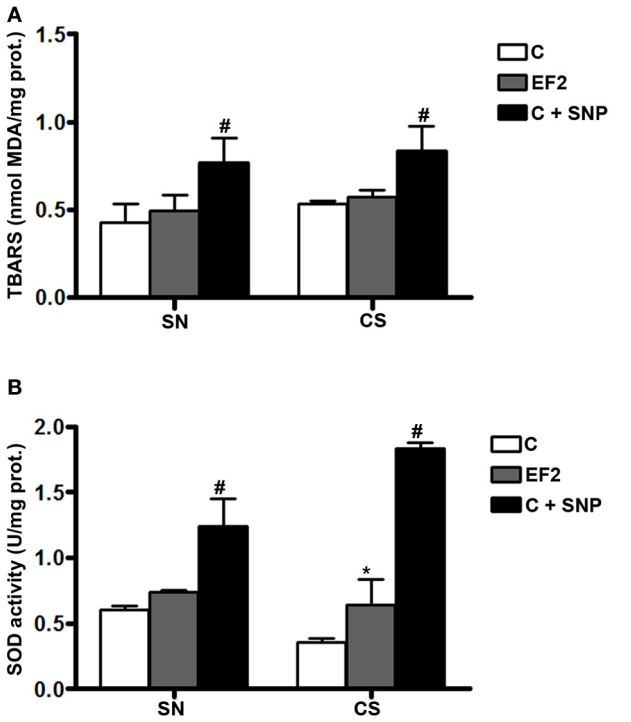

FIGURE 1 | Thiobarbituric acid-reactant substances (TBARS) levels (A) and total superoxide dismutase (t-SOD) activities (B) in the pool of Substantia Nigra and Corpus Striatum from first generation adult rats fed essential fatty acid restricted diet and controls ($n = 12$ per group). $^{*}P < 0.05$ compared 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.

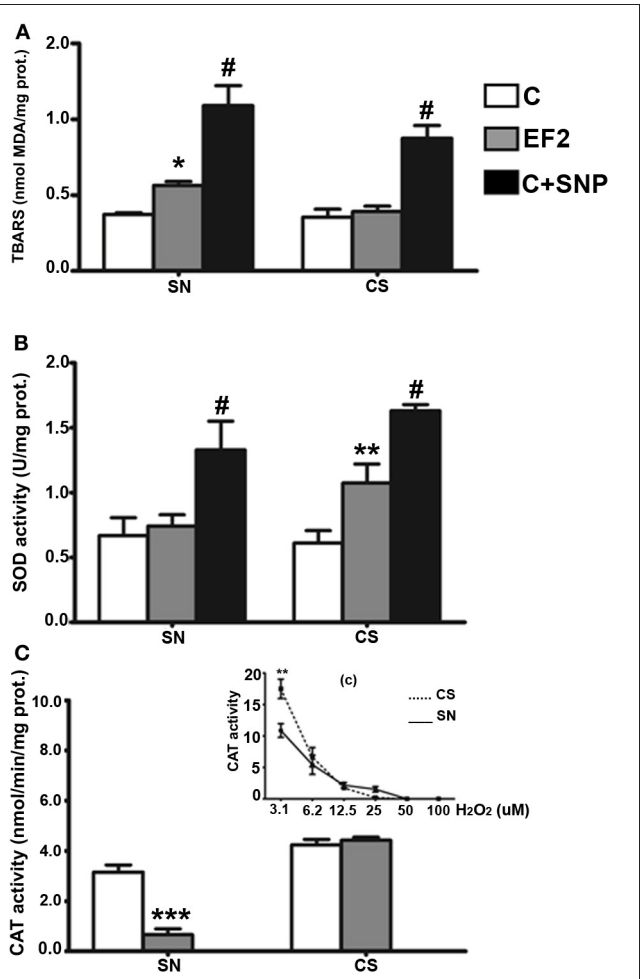

FIGURE 2 | Thiobarbituric acid-reactant substances (TBARS) levels (A), total superoxide dismutase (t-SOD) activities (B), and catalase (CAT) activities (C) in the pool of Substantia Nigra and Corpus Striatum from young rats fed an essential fatty acid restricted diet over two generations and respective 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

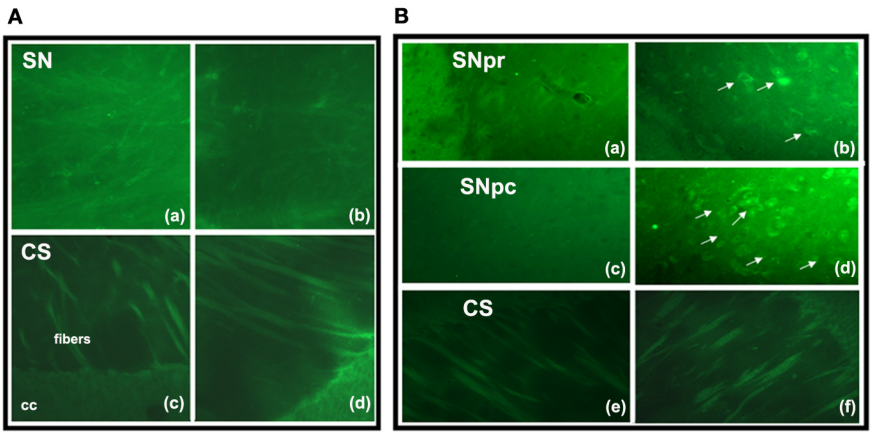

FIGURE 3 | Fluoro-Jade C staining in brain parasagittal sections of F1- (panel A) or F2- (panel B) generation groups at the level of Substantia nigra (SN) or Corpus Striatum (CS). No labeling was detected in cell bodies or processes of SN **(Aa, Ab)** and CS **(Ac, Ad)** in adult animals of F1 generation. 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.

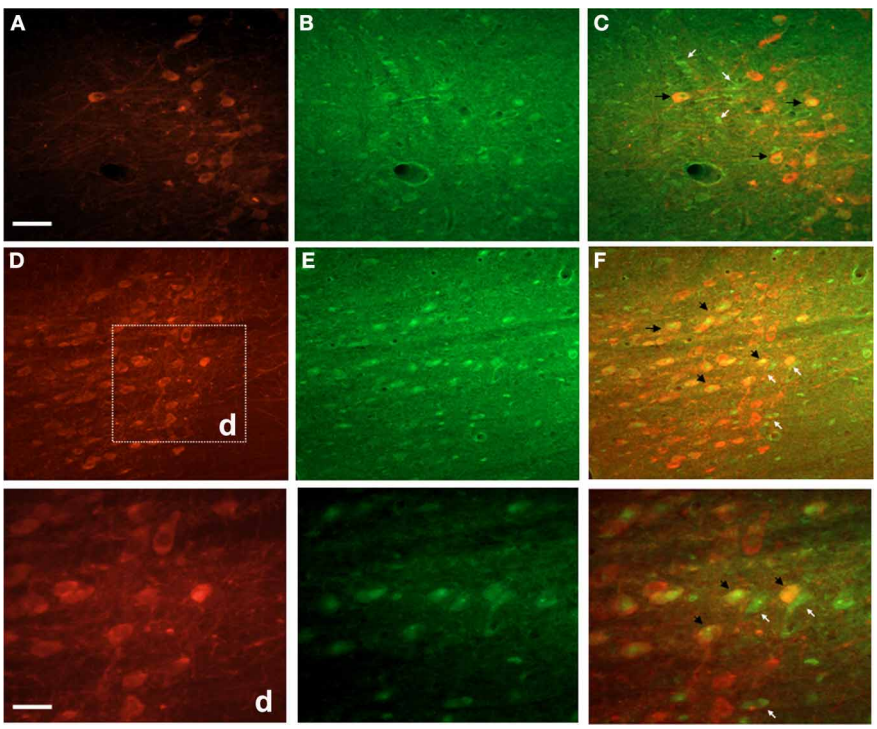

FIGURE 4 | Photographs of epifluorescence microscopy showing SN sections from a representative EF2 animal subjected to TH immunostaining followed by Fluoro-Jade C staining. Examples of single (FJC; yellow arrows) or double (TH + FJC; black arrows) labeled cells can be seen either in the SN *pars reticulata* (**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.

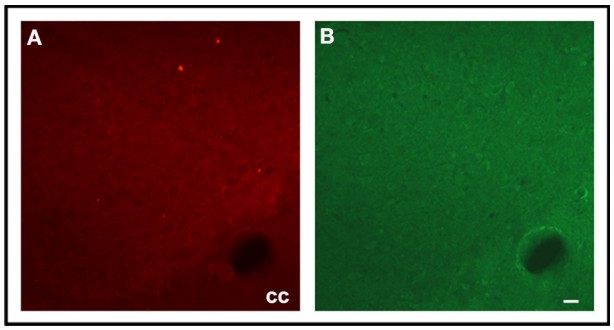

FIGURE 5 | Photographs of epifluorescence microscopy showing CS sections from a representative EF2 animal subjected to TH immunostaining (A) followed by Fluoro-Jade C staining (B). Note the absence of FJC-positive cells surrounded by TH-positive neuronal terminals. cc = corpus calosum, Scale bar = 40 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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Abstract: The vulnerability of nigro-striatal dopaminergic neurons to lesions has been discussed 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 gestation 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), 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 cell body size ($p<0.001$) and the number of TH-positive cells in the rostro-dorso-medial (~26%) and caudo-ventro-lateral (~21%) 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. An increased LP ($p<0.01$) and decrease in the CAT and t-SOD activity ($p<0.001$) were detected in the CS of the experimental group. In this same group, higher levels of LP and reduced CAT activity ($p<0.001$) 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 affect the distribution of BDNF-expressing neurons in the SN and reduce resilience of the CS increasing LP associated to a high concentration of NO and reduced anti-oxidant activity. Taken together, these effects reveal

potential mechanisms that may be acting synergistically in dopaminergic neurons degeneration induced by DHA deficiency. The present data reinforce the idea that chronic DHA deficiency can modify the brain's competence to maintain suitable flexibility under challenging conditions during adult life.

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Recife, May 16th, 2013

To **Ahmet Hoke, M.D., Ph.D.**
Johns Hopkins Hospital
Baltimore, MD 21205, USA
Editor in chief of Experimental Neurology

Enclosed is an original paper entitled "Substantia nigra neurodegeneration induced by DHA deficiency involves increased nitric oxide levels in the corpus striatum of adult rats " that we would like to submit for publication in *Experimental Neurology*.

Our present findings demonstrate, for the first time that there is a differential effect of essential fatty acid dietary restriction on oxidative stress and NO production in the nigrostriatal system. Moreover, the data corroborated our initial hypothesis that an increasing DHA depletion until adulthood reduces corpus striatum resilience, anti-oxidant resources and the number of Substantia nigra BDNF-expressing neurons, worsen dopamine cell degeneration in the midbrain. The data reinforce the idea that essential fatty acid dietary restriction during brain development and maturation can modify the brain's competence to maintain suitable resilience under challenging conditions during adult life.

There is no conflict of interest in the work reported in the present manuscript. We confirm that the results are original and have not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript that is enclosed.

Finally, we would like to attest that all efforts were made to minimize the number of animals used and their suffering.

Thank you in advance,

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Highlights

1) DHA deficiency increases nitric oxide levels in the corpus striatum
2) DHA deficiency reduces BDNF-expressing neurons in the substantia nigra
3) DHA deficiency over two generations impair striatum antioxidant resources

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 has been discussed 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 (EFA) 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 rats were raised from gestation 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), 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 cell body size ($p<0.001$) and the number of TH-positive cells in the rostro-dorso-medial (~26%) and caudo-ventro-lateral (~21%) regions of SN and the total number of BDNF immunoreactive neurons (~20%). Increased NO levels were observed in the CS of both young (~0.3 fold) and adult (~1.8 fold) experimental animals while no inter-group difference was found for this parameter in the SN. An increased LP ($p<0.01$) and decrease in the CAT and t-SOD activities ($p<0.001$) were detected in the CS of the experimental group. In this same group, higher levels of LP and reduced CAT activity ($p<0.001$) 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 adulthood affects the distribution of BDNF-expressing neurons in the SN. Moreover, increased LP associated to a high concentration of NO and reduced anti-oxidant activity, reduced CS resilience. Taken together, these effects reveal potential mechanisms that may be acting synergistically in the degeneration of dopaminergic neurons induced by DHA deficiency. The present data reinforce the idea that chronic DHA deficiency can modify the brain's competence to maintain suitable flexibility under challenging conditions during adult life.

Keywords: BDNF; lipoperoxidation; DHA; malnutrition; oxidative stress; basal ganglia; 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 et al., 1938; Gonzaléz-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 et al., 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 (Gonzaléz-Hernandez et al., 2010).

The SN exhibits a high concentration of microglia (Lawson et al., 1990) and the overactivation of these cells can result in the release of cytokines and free radicals such as superoxide radicals and nitric oxide (NO) (Minghetti et al., 1999; Duncan and Hales, 2005). These bioactive molecules released from microglia have been thought to contribute to SN dopaminergic cell death induced by mitochondrial dysfunction (Madathil et al., 2013) 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 (Gonzaléz-Hernandez et al., 1997). The potential involvement of neuronal NOS (nNOS) activity in nigral cell degeneration has been discussed. Not only due to the importance of NO as a physiological modulator of cortico-striatal glutamatergic activation but also because its synthesis in the corpus striatum (CS) can be modulated by dopamine receptor subtypes D1 and D2 (Gonzaléz-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, nucleus accumbens (Delion et al., 1996; Zimmer et al., 2000; Chalon, 2006) and dopaminergic cell loss in the 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's disease, dietary DHA supplementation partially restored 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, dietary DHA supplementation was able to increase 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 (Gall et al., 1992; Abe et al. 2010) exerting also neuroprotection in the aging brain (Singh et al., 2006; Borger et al., 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; 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 are reduced in patients with Parkinson's disease (Mogi et al., 1999). Furthermore, chronic deprivation of TrkB signaling leads to selective late onset of 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 region of SN (SNcv) 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 BDNF paracrine action on SN dopaminergic and non-dopaminergic cells, we also analyzed how the number of SN BDNF-expressing cells could be affected by EFA dietary restriction.

Materials and methods

Animals, diets and tissue processing

All procedures were approved by the Ethics Committee for Animal Research of Federal University of Pernambuco (protocol # 009428/ 200633), which complies with the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA). Adult female Wistar rats weighing 200-250 g were fed from mating throughout pregnancy and lactation on control or experimental diets, each containing approximately 400 Kcal/100g and differing only in the lipid source. The diets were prepared according to Soares et al. (1995) and met all current nutrient standards for rat pregnancy and growth as shown in Table 1. As previously described (Cardoso et al., 2012), the control diet contained 50 g/Kg of soybean oil with adequate amounts of saturated (26.01%), monounsaturated (12.32%), α-linolenic (6.04%) and linoleic (55.36%) acids. The experimental diet contained 50 g/Kg of coconut oil (from Babaçu, *Orbignya martiana*) with reduced levels of linoleic (8.10 %) and α –linolenic (0.49%) acids and higher levels of saturated (62.29%) and monounsaturated (23.73%) fatty acids. .

Please, insert Table 1 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, to provide the second generation young animals (30-42 days old, CF2Y and EF2Y groups) and adult animals (90-110 days old, CF2A and EF2A groups). A total of 25 young and 87 adult animals were used in the present work. In each group, animals were randomly sampled from different litters, housed three per cage in a room maintained at 22 ± 2 °C with 67% relative air humidity and kept on a 12 h light/dark cycle (lights on at 6:00 h). Each experimental day, three or six animals per group were anesthetized with isoflurane 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 LP 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 as nitric oxide levels via nitrite measurement. An aliquot of supernatant was analyzed for total protein content using a bicinchroninic acid protein kit (Sigma-Aldrich, St. Louis, MO).

Lipid Peroxidation (LP) quantification

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 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 with different concentrations of 1,1,3,3-tetramethoxypropane solution. Control SN and CS samples were also incubated in a 30 µM sodium nitroprusside (SNP) solution for 45 min before the assay and used as positive controls for lipid peroxidation.

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, as previously described (Cardoso et al. 2012) .

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) 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 h, with a continuous infusion pump (Harvard equipment). After perfusion, the brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem (the olfactory bulb and cochleas were excluded). They were then postfixed for 2 h in the same fixative, rinsed in PB and weighed (wet weight). Subsequently, the brains were cryoprotected in 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, 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) or 0.01 M citrate buffer (pH 6.0) at 60° C for 1 h. Thereafter, free-floating sections were rinsed in PB and incubated with a rabbit anti-BDNF polyclonal antibody (Santa Cruz, USA; 1:200) or a rabbit anti-TH polyclonal antibody (Chemicon, USA; 1:500) diluted in PB containing 0.3% Triton X- 100 (PBX) and 1% normal goat serum (NGS) for 24 hrs at 4º C. Sections were then incubated for 1hr in secondary antiserum (biotinylated goat anti-rabbit IgG; Vec-

tor 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 performed in order to identify the distribution of double and non-double labeled cells in the SN. BDNF-positive cells were stained using the DAB reaction and then the TH-positive neurons were labeled with DyLight-conjugated 488-labeled anti-rabbit IgG (1:1000, Rockland) for 24hrs. After incubation with the fluorophore-coupled antibody, the sections were washed three times in PB, mounted onto gelatin coated slides, dried at 50°C for 30 min, cleared in xylene for 1 min and mounted with Entellan (Merck). The analysis was carried out using an epifluorescence microscope (Leica, DMLB).

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 five brains per group. The quantitative analysis was performed in three (for BDNF) and four (for TH) parassagital sections at the middle level of SN, from approximately 1.4 mm until 2.9 mm latero-medial stereotaxic coordinates (plates 80 to 83 from Paxinos and Watson, 1986). These sections were from two 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). All TH- or BDNF- immunoreactive cells were counted in adjacent sampling windows throughout the extension of SNrm and SNcv 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 parasagittal sections from lateral to medial levels of SN were analyzed per animal. In the SNrm or SNcv, a minimum number of 50 cells per region/animal were set to be analyzed. Thus, a total of ~300 cells were analyzed per group in the SNrm or SNcv.

Fatty acid determination in the Corpus Striatum and Midbrain

The fatty acid profiles of CS and SN 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 phospholipids were extracted and transmethylated as previously described (Cardoso et al., 2012). The fatty acid profile was 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

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 test, $\alpha<0.05$ was further utilized to determine post-hoc comparison among groups and regions. Unless stated otherwise, all values are expressed as mean ± standard deviation (SD).

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 2).

Please, insert Table 2 around here

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

Analysis of brain fatty acid profile of adult animals demonstrated that the DHA (22:6n-3) contents in phospholipids of the EF2A group was in average 7.5 ± 0.31 and 8.0 ± 0.41 % in the SN and CS respectively. These values were ~65% lower than that obtained in the respective controls (22.12 ± 0.21 and 23.25 ± 0.51% for SN and CS). The reduced levels of DHA in the EF2A groups were accompanied by a significant increase in the docosapentaenoic fatty acid (DPA; 22:5n6) contents in the SN (10.60 ± 0.46 versus 1.45 ± 0.05 in the control) and in the CS (11.30 ± 0.36 versus 1.35 ± 0.06 in the control; 2-tail t-test, $p < 0.001$). On the other hand, the values for arachidonic acid (AA; 20:4n-6) did not differ between control and experimental animals neither in the SN (14.95 ± 0.30 versus 15.25 ± 0.35 in the control) nor in the CS (13.55 ± 0.40 versus 14.14 ± 0.55 in the control). As previously demonstrated using this experimental dietary model (Cardoso et al., 2012), regarding 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 (data not shown).

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

As shown in the Figure 1A, evidence of increased lipid peroxidation, 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 respective control group (0.4 ± 0.03 and 0.35 ± 0.02 nmol MDA / mg protein, $p< 0.05$). Thus, the magnitude of lipid peroxidation induced by the

experimental condition in the SN or CS is, respectively, 32 and 25% less than that obtained by using 30 µM SNP (1.2 ± 0.143 nmol MDA / mg protein). A significant decrease in t-SOD enzyme activity was found in the 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 EF2A and CF2A groups, respectively) (Fig. 1B). On the other hand, the CAT activity was significantly reduced in the SN (2.38 ± 0.21 nmol/min/mg protein) and especially in the CS (3.40 ± 0.60 nmol/min/mg protein) of the EF2A group compared to control groups (5.20 ± 1.11 and 9.58 ± 1.37 nmol/min/mg protein for SN and CS respectively; $p< 0.001$; Fig. 1C).

Please, insert Figure 1 around here

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

Figure 2 shows the results obtained in the SN and CS homogenates of F2 young (Fig.2A) and adult (Fig.2B) animals. In the SN, no intergroup difference in the nitrite levels was observed either in young or in adult animals. However, in the CS of both young and adult EF2 groups, nitrite levels were, respectively, 0.3-fold and 1.8-fold higher than those of the control group ($p< 0.001$). It is worth noting that in the CS of the CF2A group, nitrite levels were estimated as about 3-fold higher than those in the SN of the same group.

Please, insert Figure 2 around here

EFA dietary restriction over two generations until adulthood reduces number of TH positive neurons at the middle level of both SNrm and SNcv

Panel A in Figure 3 shows low magnification images of representative TH-immunoreactive parasagittal sections through mid- level of 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 total number of TH-immunoreactive cells in four brain parasagittal sections through latero-medial extent of control SNrm and SNcv was 623 ± 37 and 322 ± 52 cells, respectively. A comparative analysis between the two groups showed that the number of TH-immunoreactive cells in adult rats fed the deficient diet was 464 ± 34 and 255 ± 30 cells in the SNrm and SNcv, respectively, which corresponds to ~26% ($p< 0.001$) and 21% ($p< 0.05$) fewer cells than in those fed the control diet. Figure 3B

shows contrast indices for average number of TH-immunoreactive cells obtained in the SNrm and SNcv from adult rats fed control or experimental diet (n = 5 animals per group).

Please, insert Figure 3 around here

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

The long term dietary treatment with the experimental diet being used until adulthood resulted in lower dopaminergic cell body 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) according to 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

DHA deficiency reduces the number of BDNF positive neurons at the middle level of SN of adult animals

Figures 5a and d illustrate low magnification images of representative brain parasagittal sections throughout the SN of animals of CF2A and EF2A groups. As can be seen, BDNF-immunoreactivity is widely distributed throughout the entire extension of this nucleus. Figures 5b, c,e and f show higher magnification images of BDNF positive cells located in the SNrm (b and e) and SNcv (c and f) of both groups. Note that BDNF is present in a heterogeneous SN cell population, with respect to soma size and shape. Quantitative analysis of BDNF expressing cells performed with 5 animals per group at the middle level of SN (3 parasagittal sections per animal) showed that the number of BDNF-immunoreactive cells in rats fed the deficient diet was 20% lower (1009 ± 156.8 cells) than in those fed the control diet (1292 ± 156.6 cells; $p < 0.05$) (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 and C`; D and D`; E and E`) were detected in the SN of both groups. Thus, remaining

TH-positive cells in the SN of EF2A group comprise a heterogeneous cell population regarding BDNF expression.

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 adulthood could reduce the CS resilience observed in young animals, affecting its 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 in DHA-induced neuroprotection.

As has been previously reported for young animals (Borba et al., 2010; Passos et al., 2012; Cardoso et. Al., 2012), the long term treatment with an EFA deficient diet until adulthood 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. Such reduction seems to be independent of essential fatty acid deficiency (Hargrave et al., 2005). On the other hand, no intergroup difference was observed in the brain weight of adult animals compared to that observed in young animals (Passos et al., 2012) suggesting compensatory mechanisms during brain maturation. Accordingly, previous studies adopting a dietary deficiency specific for α-linolenic fatty acid for two or three generations did not report any difference in the brain weight between control and omega-3 deficient groups at adulthood (Ahmad et al., 2002).

EFA dietary restriction over two generations until adulthood reduces CS resilience increasing nitric oxide and lipid peroxidation levels

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 both SN and CS (~2 fold, compared to respective controls) affected also dopaminergic neurons located in the SNcv. Nevertheless, it should be noted that similar levels of DHA depletion in both SN and CS induced distinct mechanisms underlying the oxidative stress herein described, especially those involving NO production.

Nitrergic terminals have been reported to make synaptic contacts with both 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, suggesting that modifications in the synthesis or release of this bioactive substance into this nucleus were not involved in the loss of dopaminergic cells. Studies on protective or deleterious effects of NO on neuronal survival have been widely debated in the literature (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 conditions, such as Parkinson's disease, a high concentration of NO leads to dopaminergic cell death (Singh and Dikshit, 2007; Madathil et al., 2013). 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 (Gonzaléz-Hernandez et al., 1997). However, under such conditions, an increase in nNOS expression in the SN was not involved in cell degeneration in this nucleus (Gonzaléz-Hernandez et al., 1997). A modulatory action of DHA on NO production has been discussed, indicating that the dietary supplementation of this EFA can reduce the activity of NOS in some brain regions (Sarsilmaz et al., 2003**)**. 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 reduce the number of nitrergic neurons and glial cell reactivity in this nucleus. This latter point is currently under investigation.

On the other hand, in the CS, higher NO levels in EF2 young and adult animals occurred with different magnitude and conditions of homeostatic response. While a 0.3-fold rise in NO contents was detected in EF2Y animals, where the t-SOD enzyme was reactive and increased LP was not observed (Cardoso et al., 2012), a much more expressive elevation of NO production (~1.8 fold) was found in the EF2A group in a context with reduced t-SOD and CAT activities, and the LP twice as high as in the control condition. In the CS, nitrergic interneurons are involved in the corticoestriatal glutamatergic excitability and NO synthesis can be

modulated by D1 and D2 dopamine receptor subtypes (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 (Del Bel et al., 2005; Gomes et al., 2008). Moreover, under conditions of OS induced by 6-OHDA into the CS, pretreatment with a NO donor worsened the dopamine cell degeneration in the SN (Di Mateo et al., 2009). A recent study has indicated reduced NO production in reactive microglia upon DHA supplementation as one potential mechanism involved in its anti-inflammatory action (Lu et al., 2010). Conversely, DHA deficiency induces microglia activation in the CS (Kuperstein et al., 2008). Considering that inducible 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 and other reactive nitrogenous species which can nitrate tyrosines of proteins modifying their structure and function, leading to cell death (Bishop et al., 2009). Thus, it is possible that the significant reduction in both t-SOD and catalase enzymatic activities observed in the CS of our omega-3 deficient animals could be partially due to this type of deleterious effect of NO on these proteins, worsening dopaminergic cell loss in the SN.

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

Gomes et al., (2008) demonstrated that dopaminergic cells located in the ventral region of the rat SN were more affected by 6-OHDA- induced OS in the CS than other SN dopaminergic cell populations. In the present study, dopamine cell loss in the SNcv was detected in EF2A group, when OS was also seen in the CS. A distinct neurochemical profile related to increased expression of genes encoding pro-inflammatory cytokines and decreased expression of several glutathione-related genes (Duke et al., 2007), has been described for this SN dopaminergic cell population. Interestingly, we did not find modifications in the number of these dopaminergic cells in EF2 young animals, compared to respective controls (Passos et al., 2012). Therefore, our findings in omega-3 deficient adult animals suggest vulnerability of this dopamine cell population to conditions of reduced redox balance, especially when CS is affected (Gomes et al., 2008).

Omega-3 fatty acids availability affects BDNF expression in the SN

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. 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 of post mortem studies in humans have indicated that loss of BDNF-expressing neurons in the SN may compromise their surviving neighbors, reducing the amount of their BDNF mRNA (Howells et al., 2000). In the mouse SN, BDNF is present in dopaminergic, GABAergic and nNOS positive neurons as well as in astrocytes (Abe et al., 2010). A positive influence of DHA supplementation 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), spinal cord (Ying et al., 2012) and in the CS (Sarsilmaz et al., 2003). 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). To our knowledge, no previous study has investigated the repercussion of DHA deficiency on BDNF expression in the SN. In the present work, we demonstrated that the number of BDNF positive cells was 20% lower in the SN of EF2A group, compared to the control. While this at first appears to be consistent with the loss of nigral dopaminergic neurons, double labeling experiments demonstrated that several surviving TH-immunoreactive cells also expressed BDNF in their soma and dendrite processes while in other remaining TH-positive cells, BDNF immunoreactivity was not detected. It has been demonstrated that BDNF expression in midbrain slice cultures can be enhanced by agonists of retinoid acid receptors (RAR) whose oral administration prevents dopaminergic cell loss induced by neuroinflammation in the SN (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). BDNF is also a direct target gene of the transcription factor Nurr1 (Volpicelli et al., 2007) which is involved in the genesis, development and function of dopaminergic cells (Jankovic et al. 2005). Thus, it is possible to speculate that the reduced number of BDNF–expressing neurons and the lower values of cell body area observed in SN dopaminergic cell populations of EF2A animals could be a consequence of diverse cellular and molecu-

lar mechanisms which alone or in combination can be impaired under conditions of chronic DHA deficiency.

Conclusion

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 reduce CS resilience, the number of SN BDNF-expressing neurons, and anti-oxidant resources worsening the SN dopamine cell loss. 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, our results reinforce the idea that EFA dietary restriction during brain development and maturation can modify 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 substance (TBARS) levels **(A),** total superoxide dismutase (t-SOD) activities **(B)** and catalase (CAT) activities **(C)** in the Substantia Nigra (SN) and Corpus Striatum (CS) from adult rats fed essential fatty acid restricted diet over two generations (EF2A) and respective controls (CF2A) (*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 an indicator of nitric oxide production in the substantia nigra (SN) and corpus striatum (CS) from adult rats fed essential fatty acid restricted diet over two generations (EF2A) and respective controls (CF2A) (*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 mid-level of Substantia Nigra from adult rats fed control (CF2A) or experimental diet (EF2A) for two generations, showing dopaminergic cells in the substantia nigra rostro-dorso-medial (SNrm) (a,c) and caudo-ventro-lateral (SNcv) (b,d) (scale bar = 30 μm).
Panel B: Contrast indices for average number of TH-immunoreactive cells obtained in four parasagittal sections through the SNrm and SNcv from CF2A and EF2A animals (n=5 animals per group). 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 soma area in the SNrm and SNcv of CF2A and EF2A 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). The experimental condition resulted in lesser average soma size in both the SNrm (~23%) and SNcv (~17%) 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 mid-level of Substantia Nigra from adult rats fed control or experimental diet. Low magnification images of CF2A (a) and EF2A (d) animals showing that BDNF-expressing cells are evenly distributed into the cytoarchitetonic limits of substantia nigra (bar = 250 μm). High magnification images of SN showing BDNF positive cells in the SNrm and SNcv from CF2A (b and c) and EF2A (e and f) animals (scale bar = 30 μm). **B.** Average number of BDNF positive neurons in the SN of CF2A and EF2A groups. Data were obtained in three parasagittal sections throughout the latero-medial extension of SNrm and SNcv and express mean ± SD. *p < 0.01 compared to control group.

Figure 6. Photomicrographs of epifluorescence microscopy showing SN sections immunoreactive for BDNF, stained with DAB, and for TH, visualized with DyLight-conjugated 488-IgG**.** As can be seen, examples of BDNF (thin arrows in brown or fluorescent images) or TH

(green, arrowheads) single labeled cells are detected in the SN of rats fed control (A) or EFA-deficient (B and B`) diets. Double labeled cells for BDNF and TH are seen either in the control (C and C`, thick arrow) or in experimental animals (D and D`; E and E`, thick arrows). Thus, remaining TH-positive cells in the SN of EF2A group comprise a heterogeneous cell population regarding BDNF expression.

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

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

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

Table 2: Body and Brain Weights

Groups	Body Weight (g)	Brain Weight (g)	Brain weight/body weight ratio
CF2Y	79.65 ± 14.87 (*n* = 33)	1.69 ± 0.06 (*n* = 08)	0.0210 ±0.004 (*n* = 08)
EF2Y	71.91 ± 10.09* (*n* = 40)	1.48 ± 0.12*** (*n* = 10)	0.0200 ± 0.010 (*n* = 08)
CF2A	385.46 ± 41.75 (*n* = 15)	2.05 ± 0.18 (*n* = 06)	0.0050 ± 0.004 (*n* = 08)
EF2A	338.28 ± 36.68** (*n* = 21)	1.91 ± 0.12 (*n* = 09)	0.0056 ± 0.005 (*n* = 08)

Values are expressed as Mean ± SD. Unpaired Student´s T test
* P < 0.05; ** P < 0.01; *** P < 0.001

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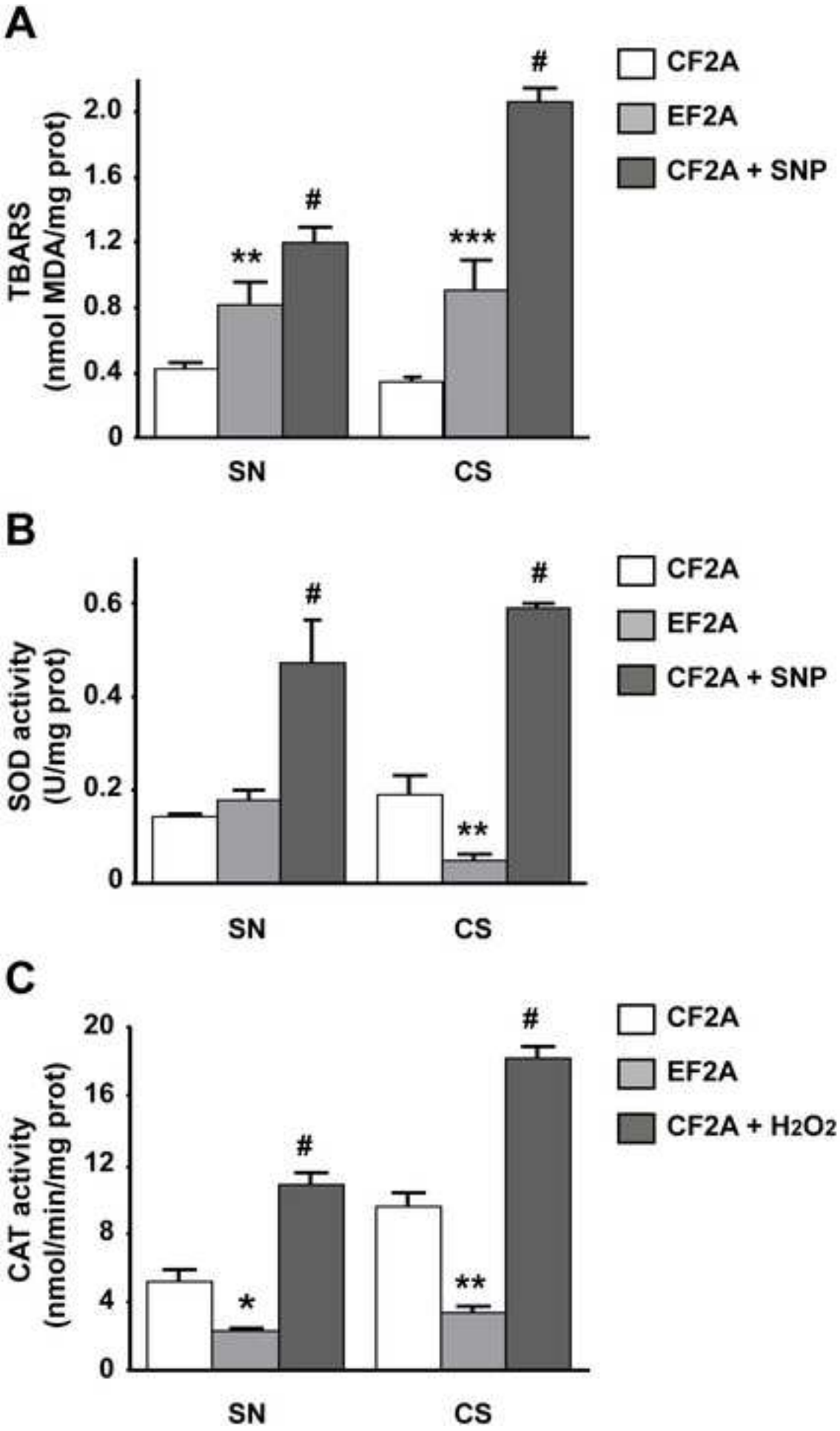

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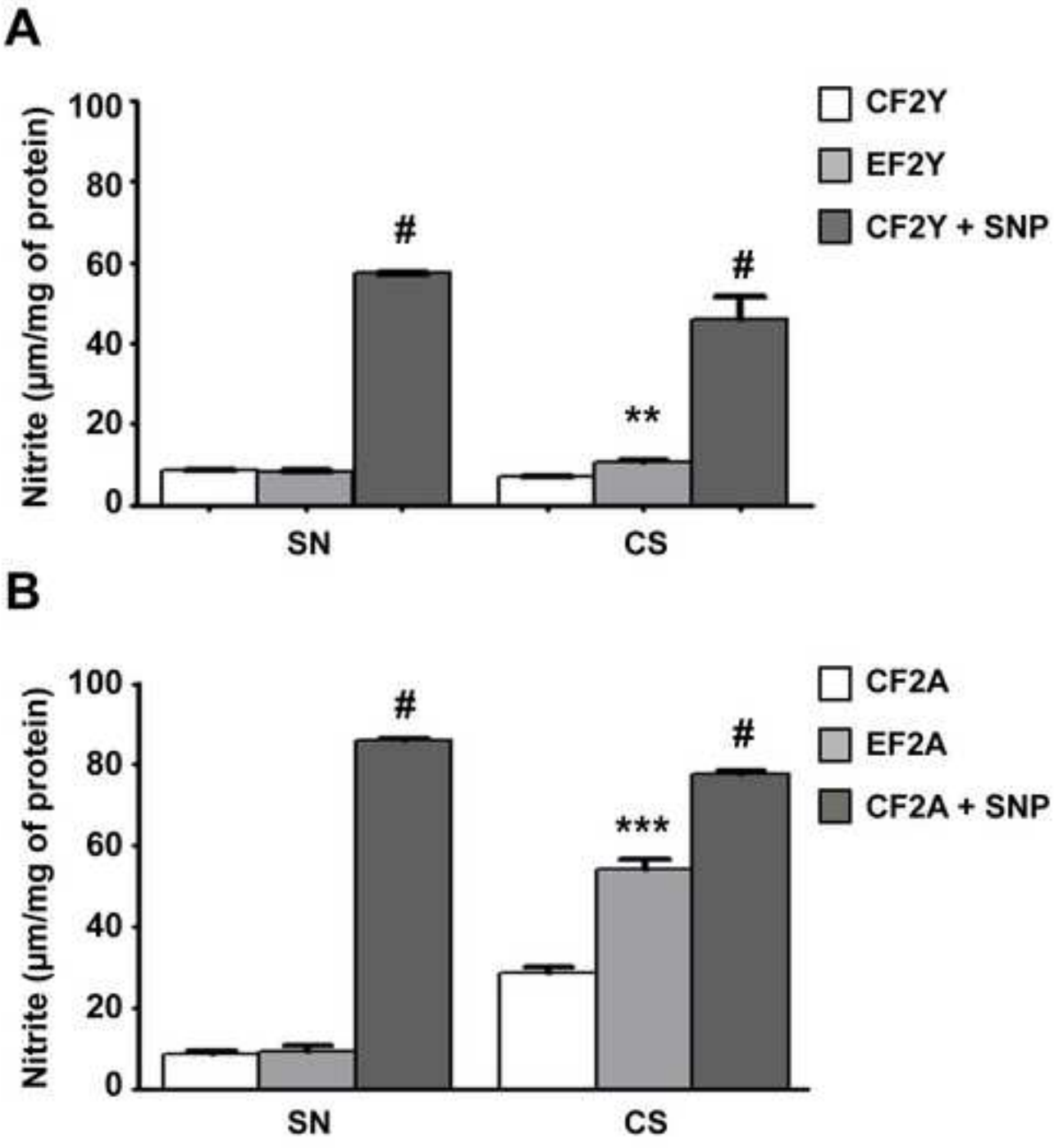

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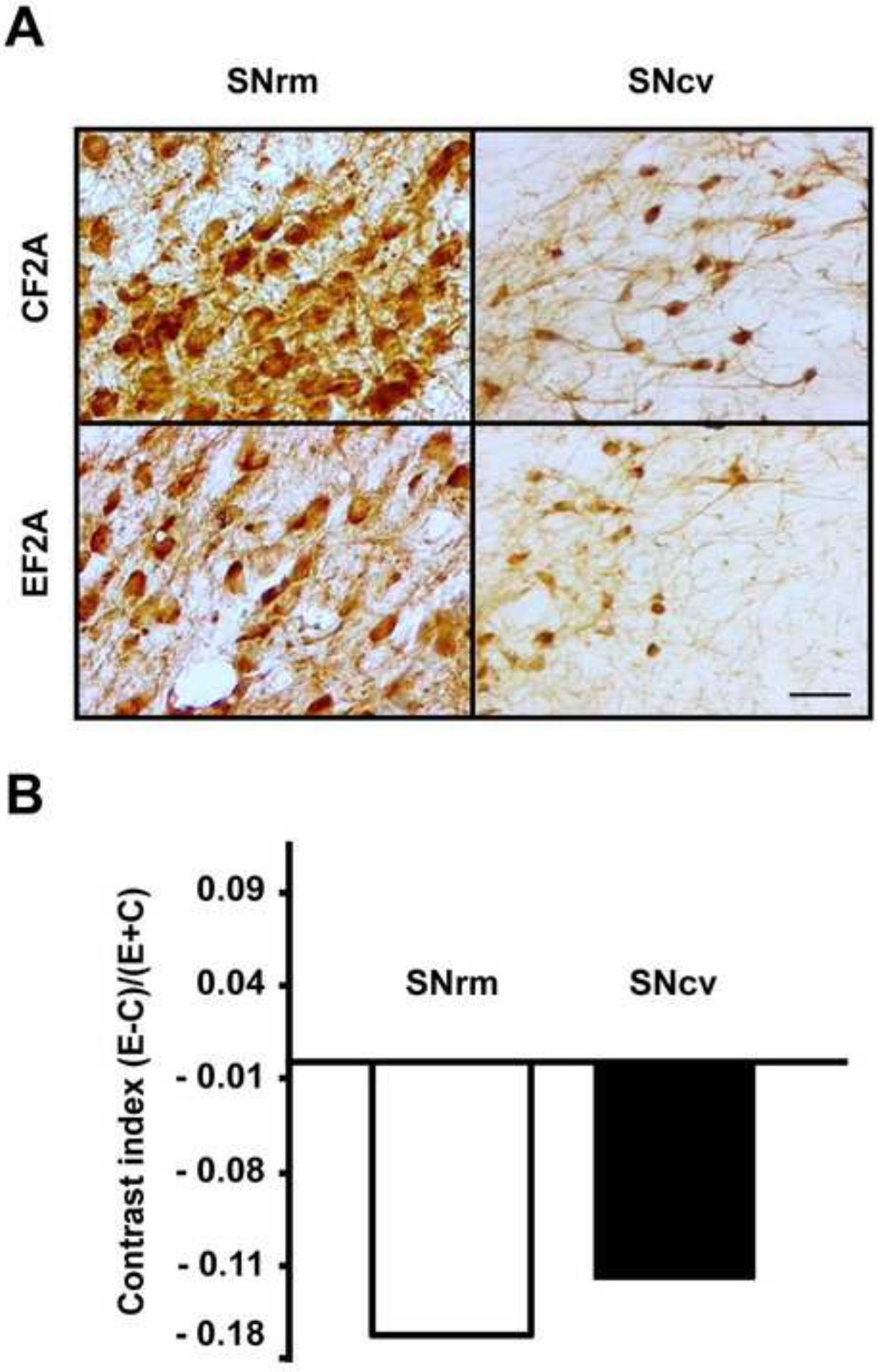

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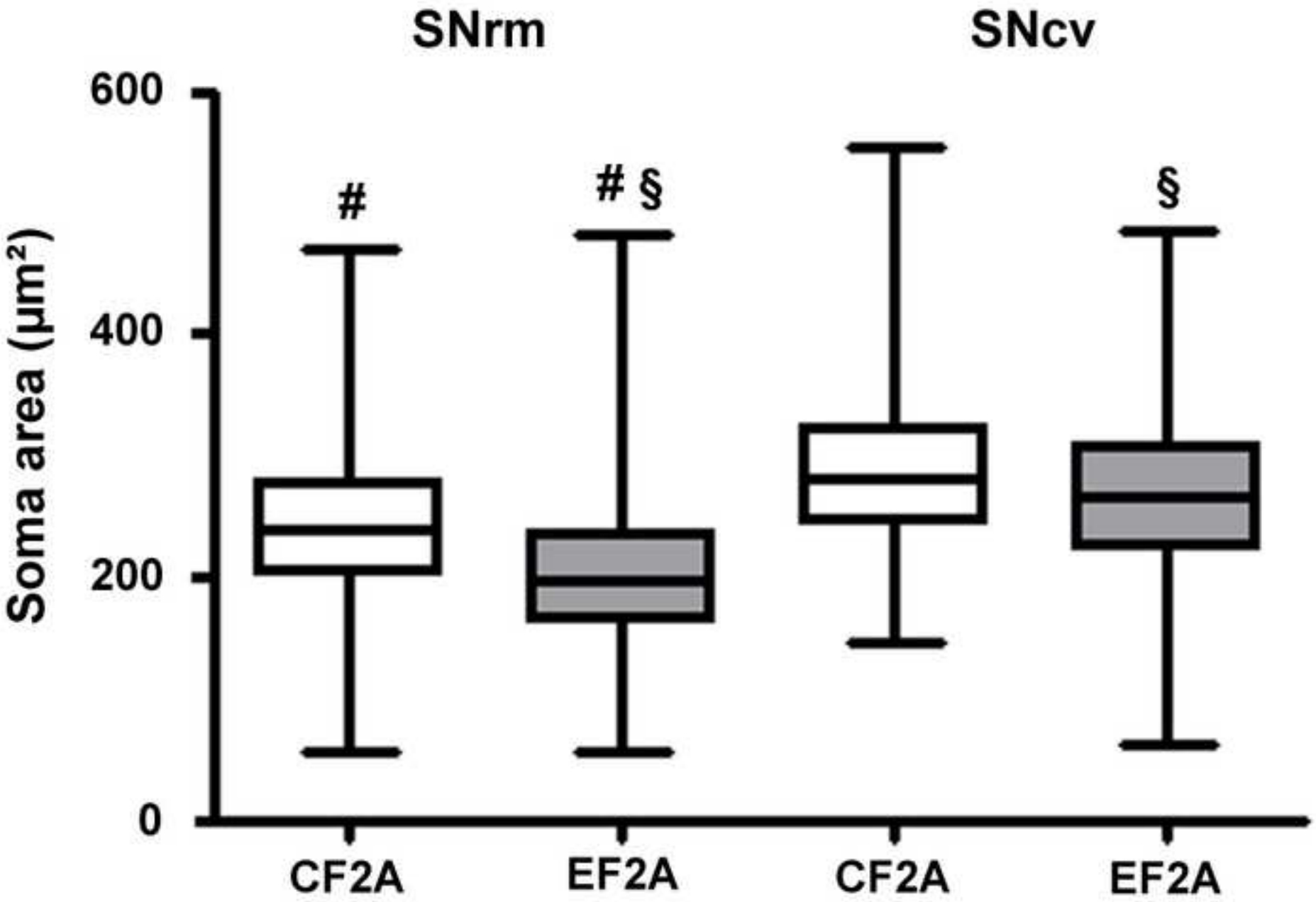

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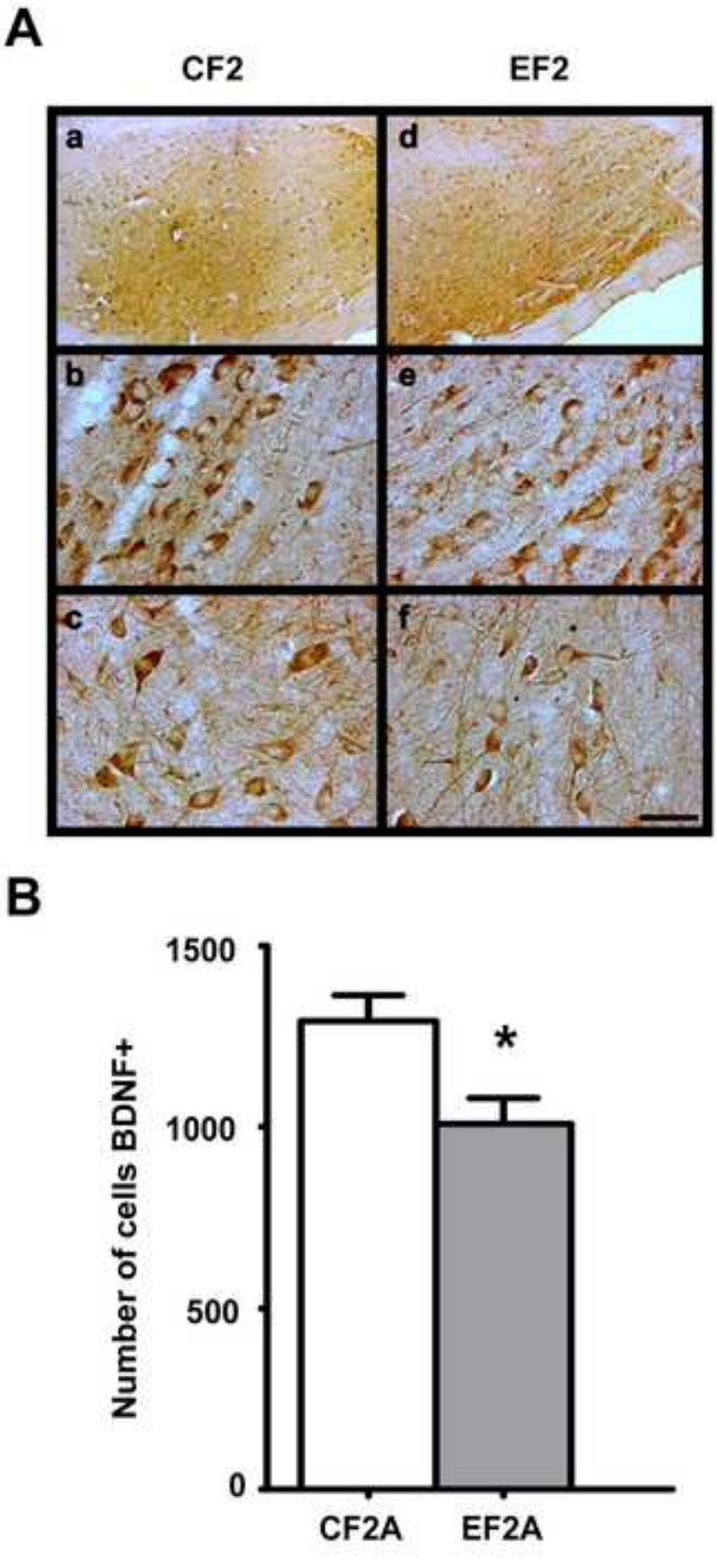

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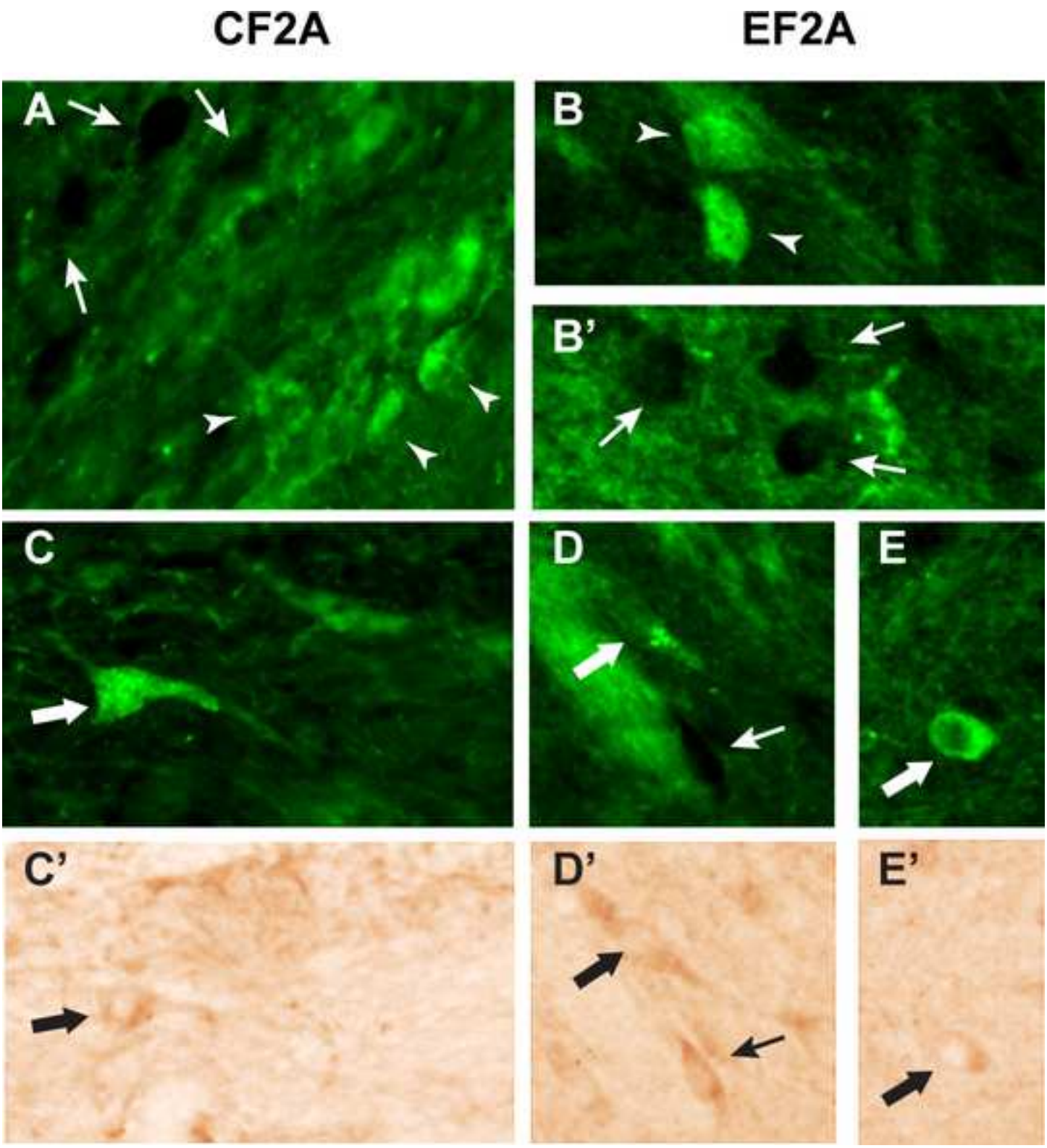

7. CONCLUSÕES

1. A restrição dietética em AGES, desde o período de gestação até a fase adulta em animais da primeira geração foi capaz de reduzir os níveis encefálicos de DHA em cerca de 28% mas ainda permitiu uma reatividade anti-oxidante capaz de evitar a peroxidação lipídica na SN e CE;

2. Nos animais jovens da segunda geração onde os nívels de depleção do DHA atingiram cerca de 50% tanto na SN como no CE, observamos uma vulnerabilidade diferenciada ao insulto oxidativo entre estas duas regiões;

3. Os dados obtidos utilizando-se FJC como marcador para neurodegeração também evidenciaram que em animais jovens submetidos à restrição dietética em AGES, além de células dopaminérgicas, outras populações neuronais presentes na SN apresentam sinais de sofrimento celular;

4. Os níveis aumentados de NO detectados no corpo estriado de animais jovens da segunda geração, submetidos à dieta deficiente em AGES, sugerem que o mesmo possa resultar de um mecanismo fisiológico compensatório desencadeado na ausência de estresse oxidativo neste núcleo. Já nos animais adultos, os níveis de NO possivelmente comprometeram a capacidade antioxidante deste núcleo devido a potencial interação com o ânion superóxido, já que as atividades da t-SOD e catalase estão significativamente reduzidas. Portanto, elevados níveis de óxido nítrico no corpo estriado podem contribuir com a morte de células dopaminérgicas na substancia negra apenas quando associados a presença de estresse oxidativo neste núcleo;

5. Mecanismos envolvidos na peroxidação lipídica observada na SN e CE de animais adultos podem ser distintos, considerando as diferenças observadas na reatividade da enzima superóxido dismutase e produção de óxido nítrico;

6. A restrição dietética em AGEs até a idade adulta aumentou a perda de células dopaminérgicas na substancia negra atingindo inclusive aquelas localizadas na região caudo-ventro-lateral deste núcleo. A redução significativa no número de células BDNF positivas sugere que uma redução nas ações parácrina/autócrina desta neurotrofina esteja parcialmente contribuindo para a neurodegeneração observada;

7. Tidos em conjuntos, estes dados sugerem que um nível crescente de insulto oxidativo induzido pela restrição dietética em AGES é capaz de comprometer mecanismos homeostáticos antioxidantes mesmo em regiões mais resistentes dos Núcleos da Base.

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ANEXOS

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

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