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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Animals, diets and tissue processing

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

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

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

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

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

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

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

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

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

2.2. Lipid peroxidation (LP) quantification

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

2.3. Total superoxide dismutase (t-SOD) activity

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

2.4. Catalase (CAT) activity

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

2.5. Estimation of nitrite concentration

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

2.6. Tyrosine hydroxilase (TH) and BDNF immunohistochemistry

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

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

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

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

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

2.8. Soma size of dopaminergic cells

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

2.9. Fatty acid determination in the striatum and midbrain

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

2.10. Statistical analysis

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

3. Results

3.1. Body and brain weights

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

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

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

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

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

Table 2
Body and brain weights.

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

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

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

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

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

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

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

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

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

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

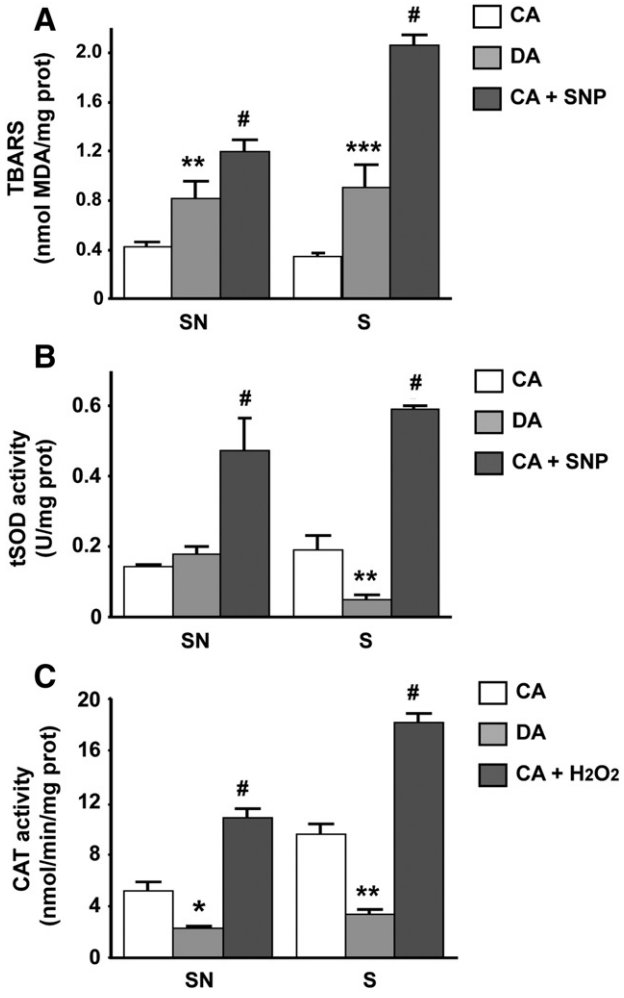

Fig. 1. Thiobarbituric acid-reactant substance (TBAR) levels (A), total superoxide dismutase (t-SOD) activities (B) and catalase (CAT) activities (C) in the substantia nigra (SN) and striatum (S) from adult rats fed EFA-restricted diet over two generations (DA) and respective controls (CA; $n = 12$ animals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

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

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

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

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

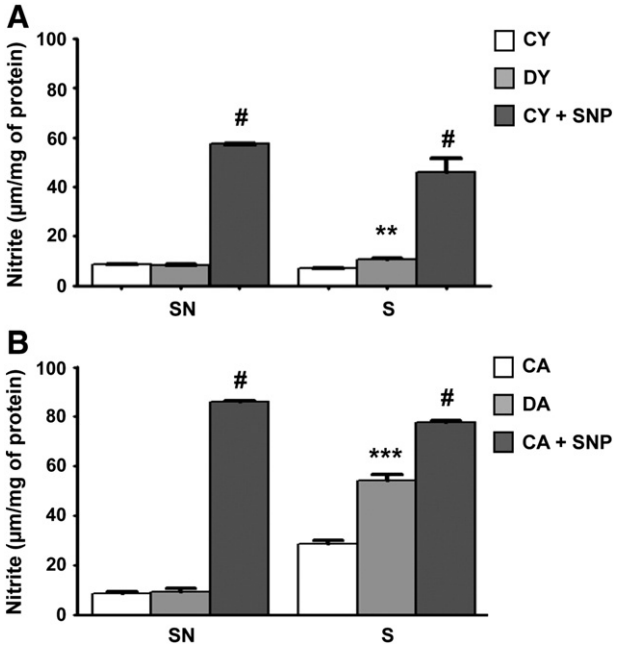

Fig. 2. Nitrite concentration as an indicator of nitric oxide production in the substantia nigra (SN) and striatum (S) from young (A) and adult (B) rats fed EFA- restricted diet over two generations (DA) and respective controls (CA; $n = 12$ animals per group). *P < 0.05; **P < 0.001 compared to control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

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

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

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

4. Discussion

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

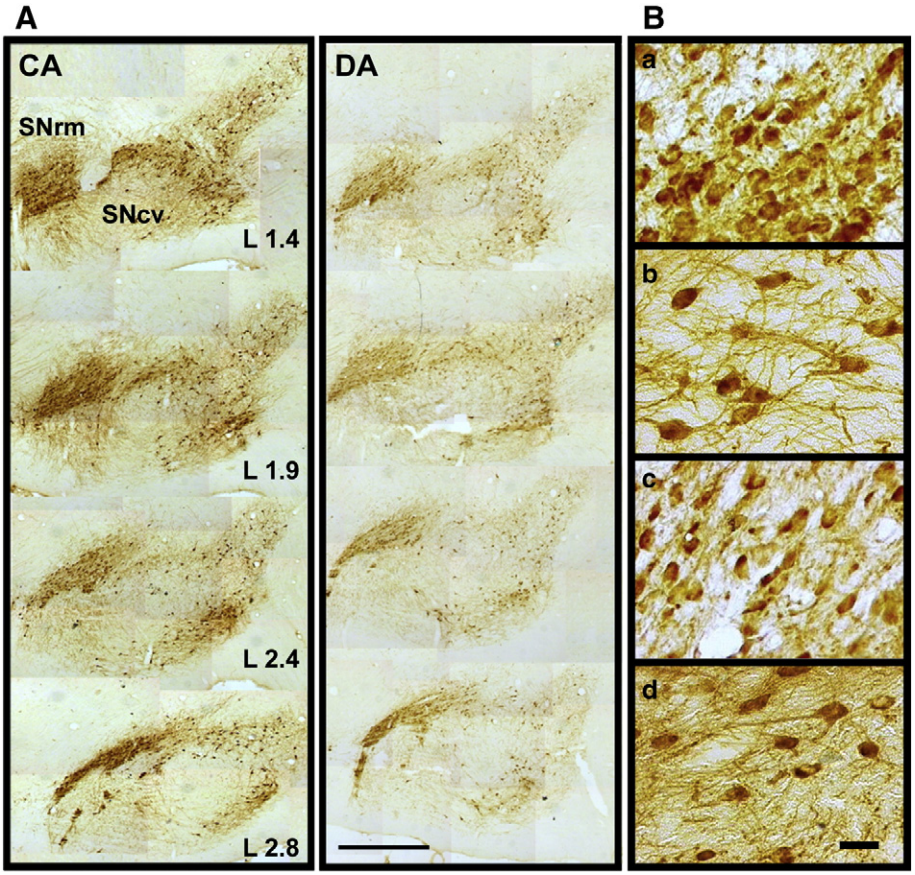

Fig. 3. (A) Representative low magnification images of TH-immunoreactive parasagittal sections though mediolateral extent of substantia nigra (SN) from adult rats fed control (CA) or EFA-restricted diet (DA) for two generations (scale bar = 500 µm; magnification power = 40×). (B) Higher magnification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

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

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

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

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

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

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

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

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

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

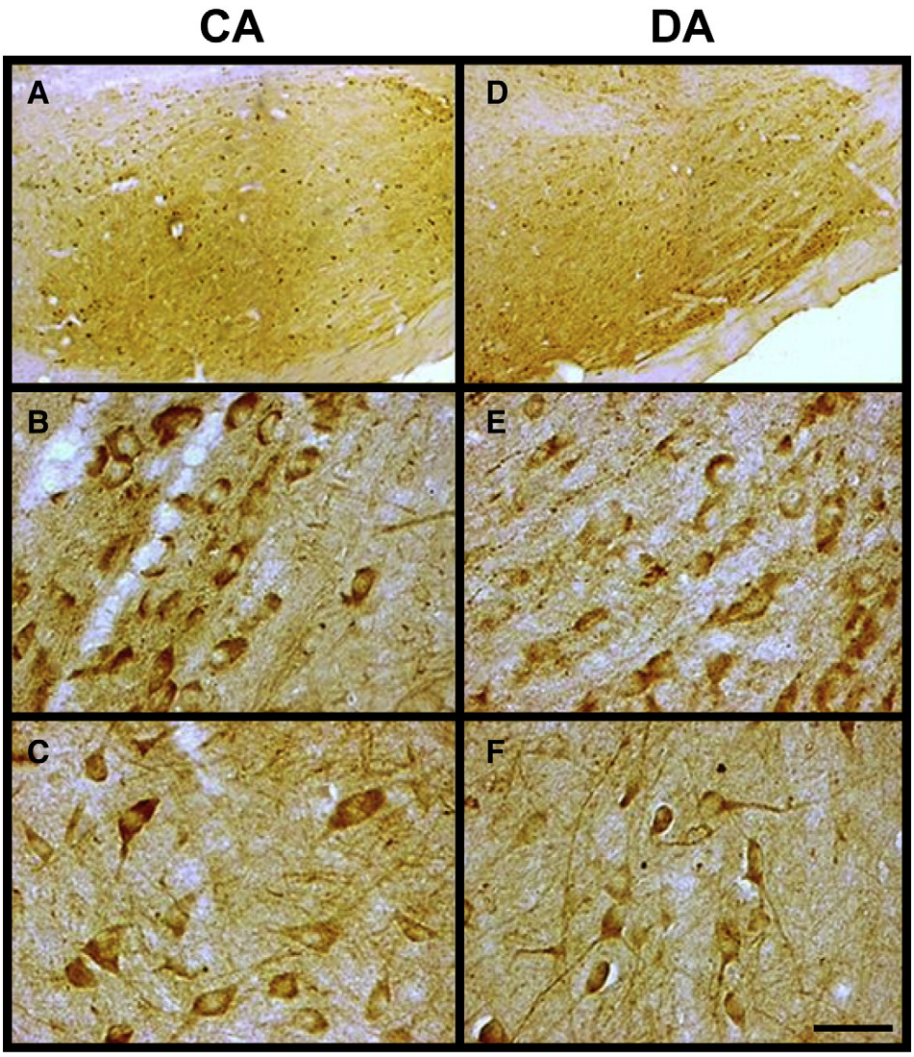

Fig. 4. Representative photomicrographs of BDNF-immunoreactive parasagittal sections at the mid-level of SN from adult rats fed control (CA) or EFA-restricted diet (DA). Low magnification images of CA (A) and DA (D) animals showing that BDNF-expressing cells are evenly distributed into the cytoarchitetonic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

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

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

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

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

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

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

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

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

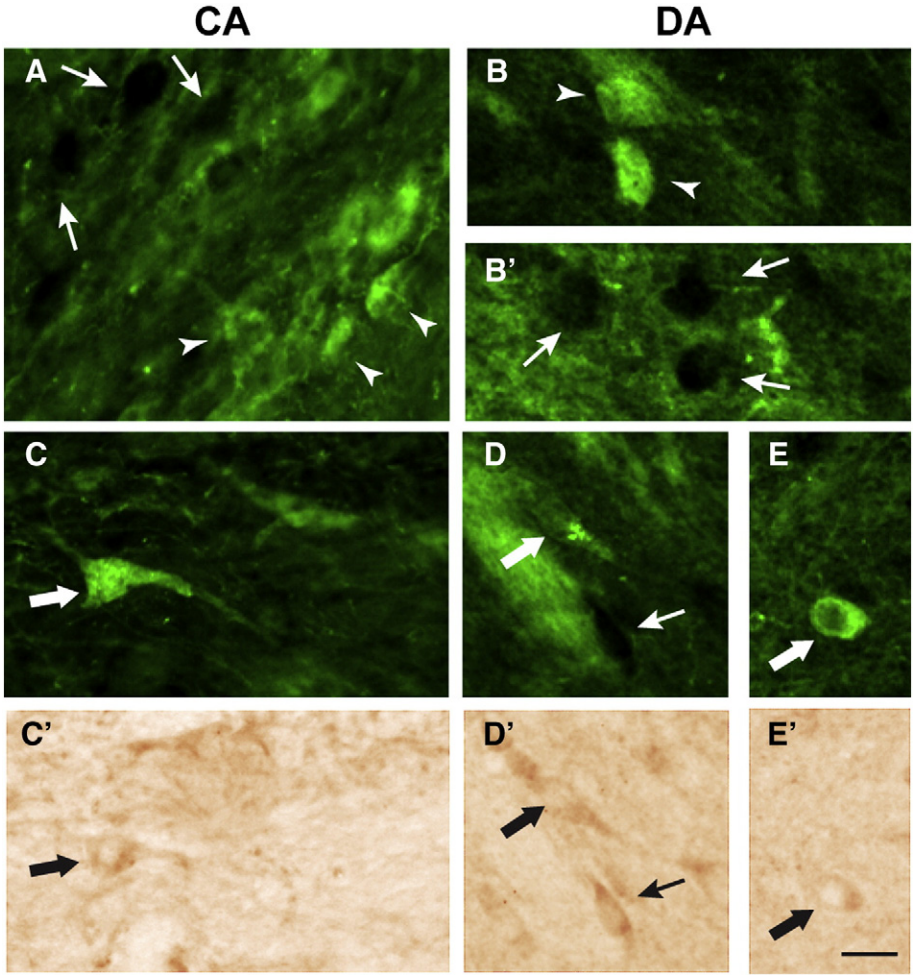

Fig. 5. Photomicrographs of epifluorescence microscopy showing SN sections immunoreactive for BDNF stained with DAB, and for TH visualized with DyLight-conjugated 488-IgG. As can be seen, examples of BDNF (thin arrows in brown or fluorescent images) or TH (green, arrowheads) single labeled cells are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

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

5. Conclusion

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

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

Conflict of interest

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

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