Dopaminergic cell populations of the rat substantia nigra are differentially affected by essential fatty acid dietary restriction over two generations

Priscila Pereira Passos^a, Juliana Maria Carrazone Borba^b, Ana Paula Rocha-de-Melo^b, Rubem Carlos Araujo Guedes^b, Reginaldo Pereira da Silva^a, Waldenício Teixeira Melo Filho^a, Kátia Maria Marques Gouveia^c, Daniela Maria do Amaral Ferraz Navarro^d, Geanne Karla Novais Santos^d, Roseane Borner^e, Cristovam Wanderley Picanço-Diniz^e, Antonio Pereira Jr.^f, Miriam Stela Maris de Oliveira Costa^g, Marcelo Cairrão Araújo Rodrigues^a, Belmira Lara da Silveira Andrade-da-Costa^{a,*}

^a Departamento de Fisiologia e Farmacologia, Centro de Ciências Biológicas, CCB, Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brazil

^b Departamento de Nutrição, Centro de Ciências da Saúde, Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brazil

^c Setor de Fisiologia e Farmacologia, Universidade Federal de Alagoas, Instituto de Ciências Biológicas e da Saúde, Campus Universitário, Maceió, AL, Brazil

^d Departamento de Química Fundamental – Centro de Ciências Exatas e da Natureza, Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brazil

^e Laboratório de Investigação em Neurodegeneração e Infecção, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil

^f Instituto do Cérebro, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil

^g Departamento de Morfologia, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Cidade Universitária, Natal, RN, Brazil

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ABSTRACT

Essential fatty acids play a crucial role in the activity of several neurotransmission systems, especially in the monoaminergic systems involved in cognitive and motor aspects of behavior. The present study investigated whether essential fatty acid dietary restriction over two generations could differentially affect dopaminergic cell populations located in the substantia nigra rostro-dorso-medial (SNrm) or caudo-ventro-lateral (SNcv) regions which display distinct neurochemical profile and vulnerability to lesions under selected pathological conditions. Wistar rats were raised from conception on control or experimental diets containing adequate or reduced levels of linoleic and α -linolenic fatty acids, respectively. Stereological methods were used to estimate both the number and soma size of tyrosine hydroxylase (TH)-immunoreactive neurons in the SNrm and SNcv. TH protein levels were assessed with Western blots. Long-term treatment with the experimental diet modified the fatty acid profile of midbrain phospholipids and significantly decreased TH protein levels in the ventral midbrain (3 fold), the number of TH-positive cells in the SNrm (~20%) and the soma size of these neurons in both SNrm (~20%) and SNcv (~10%). The results demonstrate for the first time a differential sensitivity of two substantia nigra dopaminergic cell populations to unbalanced levels of essential fatty acids, indicating a higher vulnerability of SNrm to the harmful effects induced by docosahexaenoic acid brain deficiency.

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1. Introduction

The nervous system is predominantly composed of lipids containing saturated, monounsaturated and polyunsaturated fatty acids. It is well established that balanced dietary levels of these fatty acids are necessary for the structural, metabolic and developmental integrity of the brain (Uauy and Dangour, 2006; Innis, 2007). Long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) are major components of neuronal membrane

phospholipids and are synthesized by successive desaturation and elongation of the essential fatty acids (EFA) linoleic (LA, 18:2, n-6) and α -linolenic (LNA, 18:3, n-3) respectively, which must be provided by the diet (Schmitz and Ecker, 2008).

A growing body of evidence has indicated that several different fatty acids may act as neuroprotectors in the brain. For example, DHA has been implicated in reducing apoptosis and necrosis in different classes of neurons (Lang-Lazdunski et al., 2003; Kim et al., 2010) while AA can be a neurotrophic factor on sensory neurons (Robson et al., 2010). The saturated stearic acid (18:0) is able to protect cortical neurons against the effects of oxidative stress (Wang et al., 2007) and the monounsaturated oleic acid (18:1n9) can behave as a neurotrophic factor in primary cell cultures (Medina and Tabernero, 2002) or promote axonogenesis in the

* Corresponding author. Tel.: +55 81 21268530; fax: +55 81 21268976.

E-mail addresses: belmira@gmail.com, bl@ufpe.br (B.L.d.S. Andrade-da-Costa).

striatum during brain development (Polo-Hernández et al., 2010). LC-PUFA are functionally involved with several neurotransmission systems including the monoaminergic (Zimmer et al., 2000; Chalon, 2006; Vines et al., 2012) system. Decreased levels of DHA during brain development can affect serotonergic and dopaminergic functions in cortical or subcortical regions, modifying several aspects of behavior (Levant et al., 2004; Yavin, 2006; Chalon, 2006; Kuperstein et al., 2008; Fedorova et al., 2009; Levant et al., 2010) and contributing to the etiology of some neurological diseases (McNamara and Carlson, 2006). Thus, the dietary supplementation of LC-PUFA, especially those of the *n*-3 family, has been indicated as a therapeutic strategy for a variety of these diseases (Heinrichs, 2010). In experimental models of Parkinson disease, for example, it has been shown that the dietary supplementation of DHA or fish oil may partially restore dopaminergic neurotransmission after 6-hydroxydopamine (6-OHDA) – or 1-metil-4-fenil-1,2,3,6-tetrahydropyridine (MPTP)-induced striatal lesions (Bousquet et al., 2008; Cansev et al., 2008). On the other hand, in a model that associated successive parity with a maternal diet containing deficient levels of α -linolenic acid, a reduced number of TH-immunoreactive neurons was detected in the offspring's substantia nigra pars compacta (SNpc) at postnatal day 70 (Ahmad et al., 2008). These data suggest that an adequate *n*-3 EFA dietary supply is necessary for the survival of dopaminergic neurons, at least from the SNpc. To our knowledge, no information is available on the vulnerability of other dopaminergic cell populations located in the substantia nigra (SN).

In the SN, dopaminergic cell populations are mainly distributed in two regions: the rostro-dorso-medial region of the SNpc (SNrm), and the caudo-ventro-lateral region (SNcv) including the SN pars reticulata and the ventrolateral region of pars compacta. Such populations differ in their ontogeny, morphological and neurochemical features (Bayer et al., 1995; González-Hernández et al., 2004; Duke et al., 2007) and their projections to corpus striatum can be segregated into distinct functional divisions (Joel and Weiner, 2000; Prensa and Parent, 2001). They also differ on their susceptibility to degeneration in Parkinson's disease in humans (Damier et al., 1999; Duke et al., 2007) and in rodent models (Rodríguez et al., 2001). According to these previous studies, dopaminergic neurons located in the SNcv are relatively more vulnerable to lesions and degenerate before the others. The distinct vulnerability of these dopaminergic populations has been ascribed to differences in their neurochemical profile, particularly the expression of calcium binding proteins, (Yamada et al., 1990; Gaspar et al., 1994), availability of the glial cell line-derived neurotrophic factor (GDNF) from the striatum (Barroso-Chinea et al., 2005), levels of plasma membrane dopamine transporter (Uhl et al., 1994; González-Hernández et al., 2004), increased expression of genes encoding pro-inflammatory cytokines and decreased expression of several glutathione-related genes (Duke et al., 2007).

Considering these neurochemical differences, the present study investigated whether the dopaminergic cells of the SNcv would be more vulnerable than those from the SNrm to the effects of a diet commonly used to induce EFA deficiency, due its reduced levels of both linoleic and α -linolenic acids and high contents of saturated fatty acids (Deuel et al., 1954; Hargrave et al., 2005; Ling et al., 2010). We also investigated whether this dietary treatment over two generations could affect the amount of the dopamine-synthesizing enzyme in the ventral midbrain.

2. Materials and methods

2.1. Animals and diets

Adult female Wistar rats weighing 200–250 g were fed from mating throughout pregnancy and lactation on a control or an experimental diet, both containing around 400 kcal/100 g and differing only in the lipid source. The composition of the diets and their fatty acids content are shown in Tables 1 and 2, respectively. The

Table 1

Diet composition (g/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
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%): CaHPO₄ (38); K₂HPO₄ (24); CaCO₃ (18.1); NaF (0.1); NaCl (7.0); MgO (2.0); MgSO₄ 7H₂O (9.0); FeSO₄ 7H₂O (0.7); ZnSO₄ H₂O (0.5); MnSO₄ H₂O (0.5); CuSO₄ 5H₂O (0.1); Al₂(SO₄)₃ K₂SO₄ 24H₂O (0.02); Na₂SeO₃ 5H₂O (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.

diets were prepared according to Soares et al. (1995), submitted to transesterification according to Hartman and Lago (1973) and analyzed using gas chromatography. The control diet contained 5% of soybean oil with adequate amounts of saturated, monounsaturated, α -linolenic and linoleic fatty acids. The experimental diet contained 5% coconut oil with reduced levels of linoleic acid (~7 times reduction, corresponding to about 30% of recommended minimal dietary requirement for rodents (Bourre et al., 1990)) and α -linolenic acid (~12 times reduction), and higher levels of saturated (2 fold) and monounsaturated (2.5 fold) fatty acids. The diets differed also regarding the linoleic/ α -linolenic acids ratio which was 9:1 for the control and 16:1 for the experimental version. They were prepared at least twice a month and stored in a refrigerator at 4 ± 2 °C.

The offspring (*n* = 80 males) of both groups were the object of the present study. At parturition, litter size, total litter weight and mean birth weight of the pups was recorded. Litters were then reduced to 6 pups each, by keeping the median weighted animals (identified with distinct marks). Dams and male pups were divided into two main groups according to the nutritional condition: control (C) and experimental rats (E). After weaning, on postnatal day (P) 21, the rat pups were

separated and fed *ad libitum* the same diet as their respective mothers. They were the first generation (CF1 and EF1) and were allowed to mate at 90–110 days 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 $22 \pm 2^\circ\text{C}$ with 67% relative air humidity and kept on a 12 h light/dark cycle (lights on 6:00 h). All animal procedures were carried out in accordance with the norms of the Ethics Committee for Animal Research of Federal University of Pernambuco (CEEA) which specifically approved this study (protocol # 009428/200633), and complies with the "Principles of Laboratory Animal Care" (NIH, Bethesda, USA).

2.2. Fatty acid determination in the midbrain and cerebral cortex

The fatty acid profiles of midbrain and cerebral cortex phospholipids were assessed in F2 groups at 35 days of age. Pups were decapitated and the regions containing the midbrain or cerebral cortex were rapidly dissected in an ice bath. Tissues were homogenized in a 50 mM Tris–HCl buffer with EGTA and centrifuged for 30 min at $28,000 \times g$ at 4°C . Pellets were immediately resuspended in 50 mM Tris. Total lipids of brain homogenates were extracted according to Folch et al. (1957). Phospholipids were then separated by means of a Sep-Pak procedure (Juaneida and Rocquelin, 1985) and transmethylated (Berry et al., 1965). Samples were analyzed using a Thermo Trace Ultra GC apparatus equipped with a flame ionization detector and HP-20 (carbowax 20 M) capillary column ($25 \text{ m} \times 0.32 \text{ mm} \times 0.3 \mu\text{m}$). The column temperature was initially 40°C for 1 min, then increased to 150°C by $55^\circ\text{C}/\text{min}$, and finally increased to 220°C by $1.7^\circ\text{C}/\text{min}$. The injector and detector temperatures were 200 and 220°C , respectively. Nitrogen was used as the carrier gas at a flow rate of 1.0 ml/min; injection was in split mode (1:20) and the injection volume was 1.0 μl of sample isooctane extract. A standard fatty acid methyl ester mixture (Supelco, USA) was used to identify most of fatty acid methyl esters by their retention time. The docosapentanoic acid (DPA) of our samples was identified by using gas chromatography–mass spectrometry (GC–MS). Thus, the presence of DPA peak and its mass spectrum was compared with that obtained in the mass spectrum libraries of the equipment (Willey, Nist and Adams). Fatty acid data were expressed as % of total peak area. Data are expressed as the mean $\pm$ standard error of the mean (SEM). Differences between groups were analyzed by Student's *T* test. Differences were considered significant at $P < 0.05$.

2.3. Protein extraction and Western blotting analysis

Ventral midbrain homogenates containing samples of 6 animals per group were obtained at P35–42 from the CF2 and EF2 groups. Such samples were homogenized in freshly prepared 20 mM Tris/HCl buffer (pH 7.4) containing 10 mM MgCl_2 , 0.6 mM CaCl_2 , 0.5 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 $\mu\text{g}/\text{ml}$ leupeptin and 0.05% Triton X-100. An aliquot was taken to determine protein content. An equal volume of sample buffer (62.5 mM Tris/HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% mercaptoethanol and 0.002% bromophenol blue) was added, and samples were boiled for approximately 3 min. Fractioning of protein samples was achieved using 10% polyacrylamide gels containing 0.1% SDS. After separation, the protein bands were transferred from the gel onto nitrocellulose paper, as described by Towbin et al. (1979). The nitrocellulose blots were incubated with mouse anti-TH polyclonal antibody (1:500; Chemicon, Temecula, CA, USA) or mouse anti- β -actin polyclonal antibody (1:5000; Chemicon, Temecula, CA, USA) and then incubated for 3 h at room temperature. They were subsequently exposed to mouse secondary antibodies conjugated to horseradish peroxidase and developed with a 0.16% solution of 3-amino-9-ethylcarbazole in 50 mM sodium acetate buffer (pH 5) containing 0.05% Tween-20 and 0.03% H_2O_2 . Digital images of the blots were obtained and the integrated optical density was estimated by using Labworks software (UVP Products, CA). TH protein levels were normalized to that of the β -actin protein that was used as an internal standard.

2.4. Immunohistochemical procedures

Rats (seven animals per group) were anesthetized with sodium pentobarbital (60–80 mg/kg, i.p.) and perfused transcardially first with saline (0.9% NaCl; 50–100 ml) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 (200 ml). Perfusion was always performed between 12:00 and 18:00, with a continuous infusion pump (Harvard equipment) through a cannula inserted into the left ventricle with a perfusion rate at 7.64 ml/min. After perfusion, the brains were dissected starting from the prefrontal cortex back to the inferior limit of the brainstem (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 freezing microtome (Leitz Wetzlar) into 50 μm -thick sections in the parasagittal plane, throughout the latero-medial extent of each hemi-brain. All sections were collected serially in PB and arranged in six series. The atlas of Paxinos and Watson (1986) was used to delimit the cytoarchitectonic regions. The series of sections used for immunohistochemistry

was first treated with 0.01 M citrate buffer, pH 6.0, at 60°C for 2 h. Thereafter, free-floating sections were rinsed in PB several times and incubated with rabbit anti-TH polyclonal antibody (Chemicon, USA) diluted 1:500 in PB containing 0.3% Triton X-100 (PBX) and 1% normal goat serum (NGS) for 24 h at 4°C . Sections were then incubated for 1.5 h in secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs, Burlingame, CA) diluted 1:200 in PBX, and processed for immunoperoxidase staining using the avidin biotin peroxidase complex (Standard ABC kit, Vector Labs). The binding of antibodies was revealed by the addition of diaminobenzidine tetrahydrochloride 0.05% (Sigma) and 0.01% H_2O_2 in PB, for 10 min. Subsequently, the free-floating sections were rinsed in PB and mounted on gelatin-coated slides. These procedures were carried out simultaneously in brain sections from both control and experimental animals. For the control of the staining specificity, some sections were subjected to an immunohistochemical procedure, omitting the primary antibody.

2.5. Stereological and morphometric analysis

Total estimates of the number of dopaminergic neurons in the SNrm and SNcv were obtained from seven brains per group processed for TH immunohistochemistry. In order to encompass the full latero-medial extent of the SN in the left side of the brain, six sections from a 1:6 series were analyzed for each brain. 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 dopaminergic populations were outlined with reference to an atlas of the rat brain (Paxinos and Watson, 1986). Boundaries separating the SNrm and SNcv were identified based on the location of anatomical landmarks and digitized directly from each section using a $4\times$ objective. Every sixth section containing the regions of interest was selected from a random initial sort to ensure random overall sampling. The stereology was performed at high magnification with an $100\times/1.4$ aperture oil immersion lens which allows for clear visualization of the nucleus and precise definition of the cell walls.

Pilot studies were carried out to determine suitable counting frame and sampling grid dimensions prior to counting in each region of SN to allow approximately 25–30 and 70–85 sampling sites per section for SNrm and SNcv respectively. The level of acceptable error in the stereological estimations was defined by the ratio between the intrinsic error introduced by the methodology and the coefficient of the variation. The coefficient of error (CE) expresses the accuracy of the cell number estimates and a value of $\text{CE} \leq 0.10$ was deemed appropriate for the present study, because variance introduced by the estimation procedure contributes little to the observed group variance (Glaser and Wilson, 1998). The optical fractionator method determines the number of cells by multiplying the number of objects identified inside each counting box by the values of three ratios: (a) the ratio between the number of sections sampled and the total number of sections (section sampling fraction, ssf); (b) the ratio of the counting box and the area of the grid (area sampling fraction, asf); and (c) the ratio between the height of the counting frame and the section thickness after histological procedures (thickness sampling fraction, tsf) (West, 2002). The total number of TH-positive cells was obtained using the following equation: $N = \sum Q \times (1/\text{ssf}) \times (1/\text{asf}) \times (1/\text{tsf})$ where N is the total number of cells and $\sum Q$ is the number of counted objects (West, 2002). The experimental parameters adopted for the stereological analysis are shown in Table 3.

For the soma size the area measurements were carried out using the NeuroLucida System for Neuroanatomical Analysis (MicroBrightField Inc.; Williston, USA). A systematic random sampling of cells was implemented using high magnification images with $100\times/1.3$ aperture oil immersion lenses in those cases in which the cell nucleus could be clearly identified. This analysis was carried out in six animals per group. Ten cells per section were analyzed in each region, corresponding to a total of 60 cells per region. Thus, a total of 360 cells were analyzed per group in the SNrm or SNcv. The nonparametric Kruskal–Wallis ANOVA Ranks test, $\alpha < 0.05$, 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 using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

Fig. 1A illustrates the regional borders of SNrm and SNcv used for stereological quantification and Fig. 1B shows a random sampling of cells for analysis of soma area in six parasagittal sections of two representative control animals.

3. Results

3.1. Body and brain weights

Body and brain weights at 30–42 days were significantly smaller in the EF2 group as compared to the control. Nevertheless, the brain weight/body weight ratio did not differ between the groups (Table 4).

Table 3

Experimental parameters adopted for stereological estimation of total number of TH-immunoreactive neurons in the substantia nigra rostro-dorso-medial and caudo-ventro-lateral of control (CF2) and experimental (EF2) groups.

Regions and subjects	α (frame) (μm^2)	A (x,y step) (μm^2)	asf	tsf	ssf	Number of counting frames	Number of sections	Total markers counted
<i>SNrm</i>								
CF2 group								
S1	120 × 120	120 × 120	1	0.62	1/6	152	6	1018
S2	120 × 120	120 × 120	1	0.68	1/6	147	6	980
S3	120 × 120	120 × 120	1	0.71	1/6	192	6	956
S4	120 × 120	120 × 120	1	0.69	1/6	174	6	972
S5	120 × 120	120 × 120	1	0.52	1/6	150	6	1146
S6	120 × 120	120 × 120	1	0.67	1/6	151	6	972
S7	120 × 120	120 × 120	1	0.61	1/6	178	6	1118
EF2 group								
C1	120 × 120	120 × 120	1	0.70	1/6	184	6	841
C2	120 × 120	120 × 120	1	0.67	1/6	175	6	797
C3	120 × 120	120 × 120	1	0.68	1/6	153	6	867
C4	120 × 120	120 × 120	1	0.67	1/6	159	6	908
C5	120 × 120	120 × 120	1	0.71	1/6	163	6	997
C6	120 × 120	120 × 120	1	0.68	1/6	148	6	1100
C7	120 × 120	120 × 120	1	0.62	1/6	132	6	730
<i>SNcv</i>								
CF2 group								
S1	120 × 120	120 × 120	1	0.60	1/6	437	6	649
S2	120 × 120	120 × 120	1	0.70	1/6	487	6	458
S3	120 × 120	120 × 120	1	0.60	1/6	425	6	363
S4	120 × 120	120 × 120	1	0.83	1/6	502	6	370
S5	120 × 120	120 × 120	1	0.76	1/6	471	6	675
S6	120 × 120	120 × 120	1	0.68	1/6	402	6	525
S7	120 × 120	120 × 120	1	0.65	1/6	480	6	595
EF2 group								
C1	120 × 120	120 × 120	1	0.55	1/6	421	6	361
C2	120 × 120	120 × 120	1	0.59	1/6	452	6	313
C3	120 × 120	120 × 120	1	0.76	1/6	404	6	233
C4	120 × 120	120 × 120	1	0.63	1/6	429	6	290
C5	120 × 120	120 × 120	1	0.69	1/6	455	6	419
C6	120 × 120	120 × 120	1	0.82	1/6	401	6	483
C7	120 × 120	120 × 120	1	0.88	1/6	451	6	385

All evaluations were performed using a 100× objective lens (N.A. 1.4). α (frame), area of the optical disector counting frame; A (x,y step), x and y step sizes; asf, area sampling fraction [α (frame)/A (x,y step)]; tsf, thickness sampling fraction, calculated by the height of optical disector divided by section thickness; h, section thickness; ssf, section sampling fraction.

3.2. Midbrain and cerebral cortex phospholipid fatty acid composition

Table 5 shows the mean percentage of midbrain fatty acid in rats raised on either the control or experimental diets. As can be observed, rats from the EF2 group exhibited a 50% less DHA

(22:6n-3) levels accompanied by a compensatory increase (~6 fold) in the DPA (22:5n-6) when compared to the control group. On the other hand, the values for AA (20:4n-6) did not differ between the groups. With respect to saturated and monounsaturated fatty acids, higher levels of palmitic (16:0), stearic (18:0), palmitoleic

Fig. 1. Cytoarchitectonic limits for stereological analysis. Representative schematic drawings of six parasagittal sections along the latero-medial extension of substantia nigra (4× objective) showing how the cytoarchitectonic limits of SNrm and SNcv were identified for stereologic assessment of dopaminergic cell number (panel A) and soma size (panel B). Dots represent TH positive neurons counted in each section (panel A) and ten of these neurons randomly selected per region and per section (panel B).

Table 4

Body and brain weights.

Groups	Body weight	Brain weight	Brain weight/body weight ratio
CF2	85.67 ± 14.12 g (n = 18)	1.68 ± 0.06 g (n = 12)	0.0158 ± 0.003 g (n = 12)
EF2	74.41 ± 5.90 g (n = 13)	1.56 ± 0.11 g (n = 8)	0.0148 ± 0.002 g (n = 8)

Values are expressed as mean ± SD. n, number of animals; unpaired Student's *T*-test.* *P* < 0.05.**Table 5**

Fatty acid composition (% of total) in midbrain membrane phospholipids of F2 generation groups raised on control or experimental diets.

Fatty acid	Midbrain	
	Control diet	Experimental diet
C16	16.51 ± 1.90	24.09 ± 0.27*
C16:1	0.64 ± 0.04	0.85 ± 0.03
C17	0.16 ± 0.00	0.20 ± 0.02
C18	24.88 ± 0.67	29.09 ± 0.37*
C18:1n9	16.38 ± 0.25	19.21 ± 0.21*
C18:2n6t	0.74 ± 0.17	0.65 ± 0.22
C20:1	0.62 ± 0.10	0.60 ± 0.05
C20:4n6 (AA)	13.74 ± 0.83	14.93 ± 0.48
C23	3.98 ± 0.20	3.52 ± 0.19
C22:5n6	1.49 ± 0.11	9.39 ± 0.31**
C22:6n3 (DHA)	19.70 ± 0.69	8.70 ± 0.73**

Values are expressed as means ± SD. nd = not detected.

* *P* < 0.01.** *P* < 0.001.

(16:1) and oleic (18:1n9) acids (2-tail *T*-test, *P* < 0.01) were detected in the EF2 group midbrain phospholipids in comparison to the control. Similar results were obtained in the cerebral cortex phospholipids where DHA levels in the EF2 group ($9.99 \pm 0.41\%$) were significantly lesser than that observed in the control ($21.22 \pm 0.41\%$). Increased levels of DPA were detected in the cerebral cortex of EF2 group ($9.6 \pm 0.26\%$) when compared to control ($1.54 \pm 0.06\%$) but no difference between the groups was detected

in the AA levels ($14.79 \pm 0.35\%$ and $14.93 \pm 0.43\%$ in the control and EF2 groups respectively).

3.3. Characterization and stereological analysis of SN dopaminergic cell populations

Fig. 2A and D depicts low magnification images of representative parasagittal sections through the SN in animals of CF2 and EF2 groups, respectively. The TH-immunoreactivity pattern shows the distribution of dopaminergic neurons in the SNrm and SNcv as well as in the retrorubral field (RRF). Fig. 2B, C, E, and F shows higher magnification images of SNrm (B and E) and SNcv (C and F) of both groups. As can be observed, neurons located in the SNrm or SNcv differ with respect to morphological features and soma size. TH-immunoreactivity is less intense in the EF2 animal when compared to the control.

Stereological analysis of TH-positive cells in the SN is presented in Table 6 which shows individual and average values for the seven subjects per group, including the planimetric volumes of SNrm or SNcv, coefficient of errors and biological variability. On average, the numbers of TH-immunoreactive cells in the SNrm and in the SNcv of the control group were respectively 9715 ± 1835 and 4570 ± 1510 . Combining the data of these two regions, a total of $14,285 \pm 2995$ dopaminergic neurons in the left SN were estimated. A comparative analysis between the two groups showed that in the SNrm the number of TH-immunoreactive cells in rats fed the deficient diet was 20% lower than in those fed the control

Fig. 2. Effect of EFA dietary restriction for two generations on TH-immunoreactivity in the substantia nigra. Representative photomicrographs of TH-immunoreactive parasagittal sections at the middle level of substantia nigra from rats fed control or experimental diet. Low magnification images of CF2 (A) and EF2 (D) animals showing cytoarchitectonic limits of substantia nigra rostro-dorso-medial (SNrm), caudo-ventro-lateral (SNcv) and the retrorubral field (RRF) (bar = 250 μ m). High magnification images of (A) and (D) show dopaminergic cell features in the SNrm and SNcv from CF2 (B and C) and EF2 (E and F) animals (bar = 30 μ m).

Table 6

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
<i>CF2 group</i>											
S1	13.4	1.48	0.04	6.720	0.08	12.8	0.33	0.06	9.884	0.07	16.604
S2	11.3	1.19	0.06	3.865	0.08	11.6	0.30	0.06	8.579	0.07	12.444
S3	13.2	1.07	0.05	3.617	0.08	11.2	0.24	0.11	8.180	0.07	11.797
S4	9.6	1.39	0.07	2.149	0.10	11.6	0.24	0.09	8.427	0.07	10.576
S5	10.4	1.29	0.06	5.369	0.08	15.1	0.27	0.13	13.138	0.07	18.507
S6	11.6	1.19	0.06	4.618	0.08	11.8	0.19	0.05	8.656	0.07	13.274
S7	12.4	1.32	0.05	5.650	0.07	12.9	0.36	0.05	11.141	0.06	16.791
Mean	11.7	1.28	0.055	4.570	0.081	12.4	0.276	0.060	9.715	0.068	14.285
SD	1.4	0.14	0.009	1.510	0.009	1.3	0.058	0.031	1.835	0.004	2.995
CV ²		0.012		0.109			0.044		0.035		
CE ²		0.003		0.006			0.006		0.005		
CE ² /CV ²		0.259		0.060			0.138		0.129		
CVB		0.010		0.102			0.038		0.031		
CVB ² (%CV ²)		74.02		93.98			86.36		87.07		
<i>EF2 group</i>											
C1	14.3	1.57	0.06	4433	0.09	11.4	0.33	0.08	7237	0.06	11,670
C2	13.4	1.62	0.04	3035	0.09	11.9	0.32	0.09	7122	0.07	10,157
C3	10.5	1.39	0.06	2304	0.09	11.6	0.31	0.07	7543	0.07	9847
C4	12.7	1.32	0.07	4143	0.10	11.9	0.35	0.07	8125	0.07	12,268
C5	11.6	1.44	0.06	4253	0.10	11.2	0.32	0.10	8416	0.07	12,669
C6	9.7	1.08	0.04	1722	0.09	11.7	0.33	0.08	9687	0.07	11,409
C7	9.1	1.62	0.08	2670	0.09	11.2	0.42	0.07	6158	0.07	8828
Mean	11.6	1.28	0.058	3222	0.093	11.6	0.344	0.080	7755 [#]	0.068	10,978 [#]
SD	1.9	0.14	0.015	1065	0.005	0.3	0.039	0.011	1123	0.004	1400
CV ²		0.009		0.109				0.113	0.021		
CE ²		0.003		0.008				0.006	0.005		
CE ² /CV ²		0.354		0.079				0.056	0.220		
CVB ²		0.006		0.100				0.106	0.016		
CVB ² (%CV ²)		64.63		92.11				94.33	77.99		

CE, Scheffer coefficient of error; CV, coefficient of variation; CVB, biological coefficient of variation; CVB² = CV² – CE²; SD, standard deviation.[#] *P* < 0.05 versus control.

diet (7755 ± 1123 ; 2-tail *T*-test, *P* = 0.036). On the other hand, in the SNcv no significant difference between the groups was detected (EF2 group = 3222 ± 1065 ; 2-tail *T*-test, *P* = 0.116). A total of $10,978 \pm 1400$ dopaminergic neurons was estimated in the left SN of the EF2 group which is ~23% lower than the value estimated in the respective control (2-tail *T*-test, *P* = 0.028).

3.4. Effects of the dietary treatment on dopaminergic cell soma size

In both the control and EF2 groups, the soma area of these cells ranged from 86 to 496 μm^2 in the SNrm and from 110 to 527 μm^2 in the SNcv. The soma size frequencies of these neurons were found to be distinguishable from a normal distribution (Shapiro Wilk's *W* test, *P* < 0.05) in both regions within each group. Fig. 3A and B illustrates comparative frequency histograms of soma size in the SNrm and SNcv of both groups. The predominant classes of soma size differed significantly between these two regions in each group (*P* < 0.01). The long term dietary treatment with the experimental diet resulted in lower dopaminergic cell size both in the SNrm (median = 195.89 μm^2 (84.25–514.79 μm^2)) and the SNcv (259.09 μm^2 (69.70–519.62 μm^2)) when compared to the respective controls (SNrm, median = 246.43 μm^2 (86.35–496.17 μm^2) and SNcv, median = 292.475 μm^2 (109.28–527.454 μm^2)) according to Kruskal–Wallis ANOVA Ranks test, followed by the Dunn's test, *P* < 0.01 for both regions (Fig. 3C).

3.5. Dietary EFA-deficiency over two generations decreases TH protein levels of ventral midbrain

Quantitative analysis of TH levels in homogenates of the ventral midbrain showed a pronounced decline in the expression of this enzyme in the EF2 group when compared to control. Fig. 4 (panel A) shows the single band of 62 kDa in both groups and Fig. 4 (panel

B) shows the TH/actin ratio and reveals a threefold difference between the groups.

4. Discussion

In the present study we investigated whether a long term treatment with a maternal diet containing reduced levels of both linoleic and α -linolenic acids and high contents of saturated plus monounsaturated fatty acids could affect key components of midbrain dopaminergic system and two distinct dopaminergic cell populations of substantia nigra. The results demonstrated that this multigenerational model of EFA dietary restriction changed body and brain weights, TH protein levels in the ventral midbrain and the size of dopaminergic neurons. The number of these cells in the SNrm and SNcv were differentially affected.

4.1. Repercussion of dietary treatment on body and brain weights

The smaller body weight in the EF2 group is in agreement with previous studies using coconut oil as the only source of dietary lipids for one (Deuel et al., 1954; Soares et al., 1995) or two generations (Borba et al., 2010). Regarding this effect, it has been speculated that coconut oil can reduce body weight gain due to higher fatty acid turnover rates of the saturated medium chain fatty acids (8:0–14:0) which are oxidized more rapidly than long chain fatty acids (Hargrave et al., 2005). Moreover, this effect has also been associated with dysfunction of growth hormone regulation (Soares et al., 1995). A smaller EF2 group brain weight contrasts with previous studies adopting a dietary deficiency specific for α -linolenic fatty acid for two or three generations, where no difference between the groups was detected either at 21 days (Ahmad et al., 2002a,b) or at adulthood (Ahmad et al., 2002b). Although distinct factors may be involved in brain and body weight

Fig. 3. EFA dietary restriction over two generations affects SN dopaminergic soma size. Comparative frequency histograms of dopaminergic cell soma size in the SNrm (A) and SNcv (B) of both groups show the predominant classes of soma size among the regions and groups ($N = 360$ cells per region). (C) The experimental condition resulted in lesser average soma size in both the SNrm (~20%) and SNcv (~10%) when compared to the control condition ($P < 0.01$, Kruskal–Wallis test followed by the Dunn's test). #, difference between regions; §, difference between groups.

gains in the EF2 group, the modifications herein detected were similar in magnitude and did not change the brain/body weight ratio when compared to the control group. Taken together, these data show the effectiveness of the experimental diet in inducing systemic and localized developmental effects on the rat somatic growth.

4.2. Midbrain fatty acid profile

Previous second generation studies using balanced levels of saturated but a high linoleic/ α -linolenic acid ratio (~134) have

Fig. 4. Effect of EFA dietary restriction over two generations on TH protein levels in the ventral midbrain. Panel A shows the reduction in TH-immunoreactive band of 62 kDa as compared to β -actin (42 kDa). Panel B shows the TH/actin ratio and reveals a threefold difference between the groups. Values are expressed as arbitrary units normalized to β -actin and are average of six different animals of different dams.

shown distinct levels of DHA depletion among brain regions, with midbrain being less affected (~64 reduction) than the cerebral cortex and cerebellum (~71% reduction) (Levant et al., 2006; Xiao et al., 2005). Recent evidence has indicated that a diet containing coconut oil as the only source of lipids can increase DHA depletion in the brain when compared to a fat free diet, even for a short term treatment (Ling et al., 2010). In the present study, the diet based on coconut oil and containing a linoleic/ α -linolenic acid ratio of 16 resulted in lesser DHA levels (~50%) and increased the contents of some saturated and monounsaturated fatty acids in the midbrain phospholipids. The DHA deficiency was also confirmed by the compensatory increase in the DPA levels (~6 fold) which is an specific marker of n -3 fatty acid deficiency. Similar modification on the fatty acid profile was also observed in the cerebral cortex which indicates a widespread dietary impact on other brain regions. On the other hand, AA levels did not differ between the two groups, increasing the AA/DHA ratio in both regions analyzed. We suspect that at least three factors may have contributed to these results. First, the liver has a special ability to retain AA; and sustained AA levels in the young rat brain are consistent with up-regulated conversion from linoleic acid in the maternal, fetal and newborn liver (Satomi and Matsuda, 1973; Rapoport et al., 2007). Second, potential modifications in the transport of AA across the placenta can occur (Haggarty, 2002) and third, dietary α -linolenic acid deficiency can upregulate the brain's expression of AA-selective metabolizing enzymes including cytosolic and secretory phospholipases A2 (Rao et al., 2007). Early and recent studies have also indicated 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 in other organs (Bourre et al., 1990; Brenna and Diau, 2007; Igarashi et al., 2009).

4.3. Repercussion of dietary treatment on the number of dopaminergic cell populations

Unbiased stereology demonstrated that the average total number of TH-positive cells in the SNrm of the EF2 young animals

was 20% lower than the controls. This reduction is less than that reported for the entire SN pars compacta (33%) of adult rats, associating successive parity and an α -linolenic acid dietary deficiency (Ahmad et al., 2008). Despite the choice of the EFA deficiency experimental model, altogether, these data agree with the evidence of a protective action of DHA on subsets of SN dopaminergic neurons (Bousquet et al., 2008; Cansev et al., 2008) and seems to be consistent with previous data showing modifications in terminal fields of midbrain dopamine neurotransmission or dopamine-related behavior in *n*-3 deficient rats (Delion et al., 1996; Levant et al., 2004, 2010). Moreover, the increased AA/DHA ratio (~2 fold) in the midbrain phospholipids of EF2 group could also have an effect on the magnitude of the outcomes. 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 recently reported (Tian et al., 2011). Imbalance in EFA levels, early in life, and especially *n*-3 LC-PUFA deficiency can lead to changes in brain metabolism, decreasing anti-inflammatory or anti-oxidative responses which can induce cellular suffering in different classes of neurons (Schmitz and Ecker, 2008; Innis, 2007). The increased AA/DHA ratio in the EF2 group could enhance the effects of *n*-6 fatty acids via prostaglandin synthesis (Schmitz and Ecker, 2008; Rapoport et al., 2007). It has been demonstrated that the prostaglandin PGE₂, a derivative of AA via cyclooxygenase-2 (COX-2), can induce neurotoxic effects on subpopulations of SN dopaminergic neurons in a dose and receptor-dependent manner (Carrasco et al., 2007). Furthermore, it has been also shown that dietary treatment with DHA caused a significant decrease in COX-2 activity and PGE₂ levels in mice SN, reducing MPTP-induced dopaminergic cell death (Ozsoy et al., 2011).

A smaller average number of SNcv TH-immunoreactive cells in the EF2 group was observable, but no significant difference between the groups was detected, failing to confirm our initial hypothesis that such neurons could be more vulnerable than those of SNrm to the harmful effects of EFA dietary restriction. Although future studies will have to be carried out to determine mechanisms underlying the apparent resistance of these cells in our experimental model, one aspect is of immediate interest in the discussion of this data. Midbrain dopaminergic cell populations differ regarding the availability of some neurotrophins. For example, most dopaminergic neurons of ventral tegmental area (VTA) and SNrm, but not most those of SNcv, contain GDNF retrogradely transported from striatum (Barroso-Chinea et al., 2005). Recent evidence has indicated that DHA supplementation is able to increase GDNF and neurturin (NTN) levels in the SN, reduce dopaminergic cell death induced by MPTP (Tanriover et al., 2010) as well as increase brain-derived neurotrophic factor (BDNF) mRNA levels in the striatum (Bousquet et al., 2009). GDNF, NTN and BDNF in addition to other trophic factors can play differential and synergistic roles on developing SN dopaminergic neurons, maintaining their phenotypic profile and exerting cytoprotective effects (Akerud et al., 1999; Baquet et al., 2005; Stahl et al., 2011). However, dietary *n*-3 fatty acid deficiency for a short-term or over two generations is 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 striatum (Miyazawa et al., 2010). Considering that the repercussion of LC-PUFA deficiency on the levels of some neurotrophins can differ among brain regions (Miyazawa et al., 2010), thus leads us to speculate that the differential effects on dopaminergic cell number in SNrm and SNcv could involve a distinct sensitivity of these cell populations to potential modifications on neurotrophin-related mechanisms. With respect to this, *n*-3 EFA deficiency could impair compensatory mechanisms that usually confer less vulnerability to degeneration in some midbrain dopaminergic cells. Adopting a bidimensional analysis we also detected fewer dopaminergic neurons at the middle level of VTA in

the EF2 group (Supplementary Fig. 1). Such results are qualitatively similar to that described by Ahmad et al. (2008) using stereology. Likewise the SNrm, the VTA region usually displays highest resistance to degeneration when compared to SNcv (Barroso-Chinea et al., 2005).

4.4. Repercussion of dietary treatment on dopaminergic cell size

The lower values of soma area observed in both SN dopaminergic cell populations of the EF2 group seems to be qualitatively similar to previous data on neurons of the hippocampus, hypothalamus, and both piriform and parietal cortices in multigenerational models of α -linolenic deficiency (Ahmad et al., 2002a,b). These previous studies did not identify the neurotransmitter phenotypes and showed that reduction in neuron size in the DHA-deficient diet group was greater in rats at weaning age (21 days) compared to older ones (68 days) in most brain regions, except in the piriform cortex (Ahmad et al., 2002a). In the CA1 region of hippocampus, for example, *n*-3 deficient diet induced lesser size of neurons without altering other parameters including total number of neurons (Ahmad et al., 2002b). The present data on SN dopaminergic cells of the EF2 group at 30–42 days differs from those reported in the SNpc of Long Evans rats at 70 days (Ahmad et al., 2008) where no difference on soma volume was detected between the control and *n*-3 deficient groups. Although methodological issues and age of animals may have contributed to the observed differences, the present findings reinforce previous evidence that DHA levels can affect the growth and development of neurons and suggest that this effect may not be specifically restricted to a particular neurochemical profile or brain region. With regard to this, it is noteworthy to consider that in the developing brain, DHA is a ligand for the retinoid X-receptor (Lengqvist et al., 2004), which plays a major role in the morphological differentiation of dopaminergic neurons (Castro et al., 2001).

4.5. TH protein levels in the ventral midbrain

Western blotting analysis in ventral midbrain of the EF2 group shows a lower TH protein levels when compared to the controls. Taking into account that our samples contain other TH-immunoreactive nuclei adjacent to SN, these results show a diffused effect of EFA dietary restriction on midbrain dopaminergic systems. They are also consistent with the reduced TH-immunoreactivity pattern and number of TH-immunoreactive neurons herein detected in the SN. A recent study using a diet containing ten times higher linoleic to α -linolenic acid ratio (~178) than the ratio presented in our experimental diet, reported a time-dependent decline in the TH levels of SN in lactant pups of the first generation (Kuperstein et al., 2008). The present work using a two-generation dietary treatment reinforces the sensitivity of this rate-limiting enzyme in the biosynthesis of dopamine to unbalanced levels of fatty acids, indicating a long-lasting, non-compensated effect on the ventral midbrain.

5. Conclusion

Our results demonstrate for the first time a differential sensitivity of two functionally distinct SN dopaminergic cell populations to unbalanced dietary levels of EFA. Considering that under physiological conditions SNrm is usually more resistant than SNcv to other different kinds of insults, the present results suggest that EFA dietary restriction during brain development, could impair homeostatic mechanisms, modifying the degeneration profile of dopaminergic cells under a variety of pathological conditions. In this respect, the current findings seem to be in

agreement with a recent prospective study in humans, that has positively associated a high intake of PUFA, specifically *n*-3 PUFA, with a lower risk of developing Parkinson disease (de Lau et al., 2005).

Conflict of interest

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchemneu.2012.05.003>.

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