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Partial neurorescue effects of DHA following a 6-OHDA lesion of the mouse dopaminergic system

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Abstract

Pre-clinical data collected in mouse models of Parkinson's disease (PD) support the neuroprotective potential of omega-3 polyunsaturated fatty acids (n-3 PUFA)-enriched diet on the dopaminergic (DAergic) system. In this study, we investigated the effects of an n-3 PUFA-rich diet using a neurorescue/neurorestorative paradigm. C57BL/6 adult mice were submitted to a striatal stereotaxic injection of the neurotoxin 6-hydroxydopamine (6-OHDA) to induce striatal DAergic denervation and subsequent nigral DAergic cell loss. Three weeks post-lesion, mice received either a docosahexaenoic acid (DHA)-enriched or a control diet for a period of 6 weeks. HPLC analyses revealed a 111% post-lesion increase in striatal dopamine levels in the DHA-fed animals compared to controls (ctrl, $P<0.05$), although no improvement in the motor behavior was observed. DHA treatment led to a 89% rise in tyrosine-hydroxylase (TH)-immunoreactive terminals within the striatum ($P<0.05$) in lesioned animals. Despite the fact that DHA did not change the number of TH+ neurons in the substantia nigra pars compacta (SNpc), morphological analyses revealed an increased in perimeters (+7%) and areas (+21%) of DAergic cell bodies in treated animals. Collectively, our results suggest that DHA induces a partial neurorescue/neurorestoration of the DAergic system and support further studies to investigate the potential of a diet-based intervention, or at least the combination of such approach, to current treatments in PD.

Keywords: 6-Hydroxydopamine; Neurorescue; Neurorestoration; Docosahexaenoic acid; Dopamine; Parkinson's disease

1. Introduction

A growing body of evidence collected from epidemiological, clinical and pre-clinical studies suggests that nutraceutical compounds such as omega-3 polyunsaturated fatty acids (n-3 PUFA) may have a protective effect on the development of some features associated to neurodegenerative diseases [1]. Over the last decade, a number of groups, including ours, have reported the neuroprotective action of n-3 PUFA against, in part, the dopaminergic (DAergic) neuronal degeneration characteristic of animal models of Parkinson's disease (PD) [1–4]. More specifically, we have shown that n-3 PUFA intake can blunt the neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxin used to recreate some of the pathological hallmarks of the disease in animals [2]. While the neuroprotective capacities of n-3 PUFA against the development of PD-associated pathology is important from a preventative perspective, patients afflicted by the disease are diagnosed when neurodegenerative processes are well underway [5,6]. Thus, despite the importance of demonstrating the neuroprotective effects of omega-3 in delaying the manifestation of symptoms related to neurological diseases, a critical question remains as to whether such compounds can also exert neurorescue/neurorestorative effects on the DAergic system.

Docosahexaenoic acid (DHA; 22:6 n-3, or cervonic acid) is the n-3 PUFA that is most abundant in the brain [7]. A number of mechanisms of action for its impact on neuronal function have been proposed, many of which may relate and lead to neurorescue/neurorestoration and functional recovery [3,8]. For example, DHA exhibits anti-inflammatoire and anti-oxidant properties as well as a stimulating effect on cellular signaling pathways involved in neuronal survival

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[9–13]. Higher cerebral DHA levels are also associated with increased cell body size in various neuronal populations. This has been suggested both by electrophysiology where it has been demonstrated that cells exposed to DHA have a higher cell capacitance, indicating greater total cell membrane area [14] and by immunohistochemical stainings in hippocampal neurons of animals fed with DHA-enriched diets [15]. n-3 PUFA also stimulate the secretion of brain neurotrophic factors which are known to exert neurorestorative effects [16,17].

To evaluate the potential of DHA as a neurorescue/neurorestorative agent, we exposed C57BL/6 mice to a DHA-enriched diet after the lesioning of the nigrostriatal pathway using the toxin 6-hydroxydopamine (6-OHDA). Our hypothesis was based on the idea that DHA supplementation would enhance the recovery of the DAergic system, as assessed by a range of complementary methodologies.

2. Materials and Methods

2.1. Animals

Eighty adult C57BL/6 mice (9-week-old males) were purchased from Charles River Laboratories (QC, Canada). They were housed five per cage, in ventilated cages (standard dimensions of 20 cm×32 cm×15 cm) with free access to water and fed with their respective diets (control or enriched in n-3 PUFAs, see details in sections below) for a period of 6 weeks beginning 3 weeks following the 6-OHDA lesion (Fig. 1A). Mice were further kept under a 12/12-h light/dark cycle throughout the experimental protocol. Behavioral tests were performed at three time points. The first test served as baseline before the animals were put on diet and at 3 and 6 weeks following the initiation of the protocol (Fig. 1A). All experiments were performed in accordance with the Canadian Guide for the Care and Use of Laboratory animals, and all procedures were approved by the animal research committee of the Centre de recherche du CHU de Québec.

2.2. Unilateral 6-OHDA lesion

Animals were anaesthetized using isoflurane (Sigma–Aldrich, MO, USA) and placed onto a mouse stereotactic frame (Kopf Instruments, CA, USA) as previously described [18]. Briefly, 6-OHDA (Sigma–Aldrich) was dissolved to a concentration of 2 μg/μl in 0.9% saline and 0.02% ascorbic acid, and a volume of 2 μl was injected unilaterally into the right striatum at a rate of 0.5 μl/min. The injection was performed using a Hamilton syringe at the following coordinates: AP: +0.04 cm, ML: −0.18 cm, DV: −0.31 cm (corresponding to the atlas of Paxinos and Franklin [19]). The needle was left in place for 3 min after the injection before complete retraction. Sham mice were subjected to the same surgical procedures but were injected with 2 μl of a vehicle solution (0.9% saline and 0.02% ascorbic acid).

2.3. Diets

Animals were fed with a control or an n-3 PUFA-enriched diet for 6 weeks, given between week 3 and week 9 following the 6-OHDA lesion (Fig. 1A). DHA was obtained in a microencapsulated formulation to avoid oxidation (MC60TDHA-NGH, gift from DSM, MD, USA), the dose reaching 0.5 to 1.0 g/kg/daily per animal. The formulation was produced from algae grown under tightly controlled conditions in FDA inspected facilities. It contained no oceanic pollutants and toxins such as those potentially found in fish and fish oil. Pelleted chow was subsequently generated by Research Diets Inc. (NJ, USA). Purified diet formulations were standardized to insure consistency and eliminate batch-to-batch variation. Both diets were isocaloric and contained similar concentrations of macronutrients, vitamins and minerals. Detailed descriptions of diet contents are provided in Table 1.

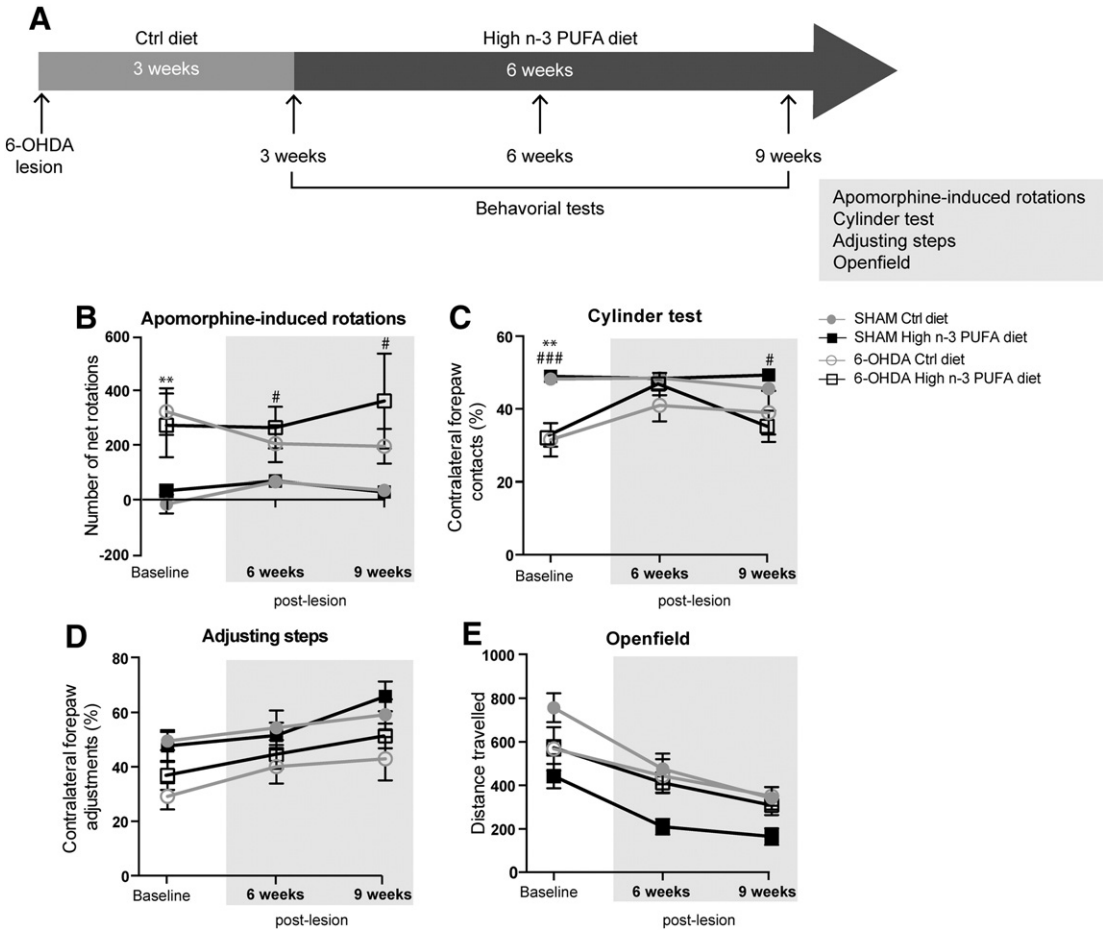

Fig. 1. **Absence of dietary n-3 PUFA effects on behavioral outcomes associated to a 6-OHDA lesion.** (A) Timeline of experimentation. (B–E) Behavioral impairments resulting from the 6-OHDA lesion remained unchanged by the intake of an enriched n-3 PUFA diet. Behavioral measures included apomorphine-induced rotations (B), cylinder test (C), adjusting steps (D) and openfield (E), and all were assessed at three distinct time points: before the beginning of the diet (3 weeks post-surgery; baseline), as well as 6 and 9 weeks post-lesion. Statistical analyses were performed using one-way ANOVA. * $P<0.05$, ** $P<0.01$ lesioned versus unlesioned mice on control diet; # $P<0.05$, ### $P<0.01$ lesioned versus unlesioned mice on n-3 PUFA-enriched diet. Values are expressed as means±S.E.M. Abbreviations: 6-OHDA: 6-hydroxydopamine; Ctrl: control; n-3 PUFA: polyunsaturated fatty acid omega-3.

2.4. Behavioral measures

Behavioral testing began 3 weeks post-lesion, before the animals were put on their specific diets, and at 6 and 9 weeks post-lesion (Fig. 1A). All tests were performed in the morning and on consecutive days (a single test per day). Mice were further separated into two distinct groups to avoid drug interference induced by the apomorphine injection (0.05 mg/kg). The first group was assigned to the cylinder test where mice were placed in a cylinder of 10 cm of diameter for a 3-min observational period. The number of contacts made by each paw against the glass walls was counted as unilateral 6-OHDA lesioned animals show forelimb asymmetry due to dopamine impairments [20]. Animals of the first group were further assigned to the adjusting steps test. Mice were held by the base of the tail with their hindlimbs suspended above the table and moved backwards at a steady rate so that they covered a distance of 1 m over a few seconds [20]. The second group of mice was assigned to the apomorphine-induced rotational test, which calculates the total number of ipsilateral and contralateral rotations (with respect to the lesion side), indicative of the extent of the lesion. These mice were further tested using the openfield which allows to quantify locomotion in an opened area. The 60-min test, yielded, in part, values on the total distance travelled [20].

2.5. Tissue preparation

Animals were sacrificed 9 weeks following the 6-OHDA lesion via an intracardiac perfusion of PBS. This was performed under deep anesthesia using a ketamine and xylazine mixture (100 mg/kg and 10 mg/kg, respectively). Brains were collected, and anterior and posterior parts were dissected. The anterior section, corresponding to structures anterior to Bregma −2.06 [19], was snapped frozen and stored at −80°C for cryostat sectioning into coronal sections of 20 μm. This method was used to specifically isolate the striatum for western blot and HPLC analyses. Additional sections of 12 μm in thickness were obtained for immunochemistry (IHC) and autoradiography analyses. The posterior part of the brain (below Bregma −2.06) was postfixed with 4% paraformaldehyde (PFA) pH 7.4 for 48 h and transferred to 20% sucrose in 0.1-M PBS for cryoprotection and cut onto a freezing microtome (Leica Microsystems Inc., ON, Canada) at 25 μm.

Table 1
Description of dietary treatments.

	Control	High n-3 PUFA
	Medium LNA	Low LNA
	No DHA	High DHA
Protein (% w/w)	20.3	20.3
Carbohydrate (% w/w)	66.0	66.0
Fat (% w/w)	5.0	5.0
Calorie per diet weight kcal/g	3.9	3.9
Ingredients (g/kg)		
Casein	200	184
DL-methionine	3	3
Corn starch	150	150
Sucrose	500	500
Cellulose, BW200	50	50
Corn oil	10	4
Safflower oil	10	17
Gelatin-coated DHA	0	45
Soybean oil	30	0
Fatty acids (g/kg) [a]		
n-3 PUFAs	1.43	7.80
Linolenic acid	1.42	0.14
Eicosapentaenoic acid	0	0.26
Microencapsulated Docosahexaeonic acid	0	7.20
n-6 PUFAs	21.20	18.42
Linoleic acid	21.10	16.65
Arachidonic acid	0	0,06
n-6 PUFAs:n-3PUFAs	14.82	2.36

Abbreviations and notes:

- n-3 and n-6 = n-3 and n-6 polyunsaturated fatty acids (PUFA).
- DHA: Docosahexaenoic acid (22:6n-3) (from high-DHA microencapsulated powder (Ocean Nutrition Inc)).
- This formulation contains at least 5:60 ratio of EPA to DHA.
- LNA: Linolenic acid (18:2 n-3) (from soybean oil).
- Proteins are from casein and DL-methionine, carbohydrates are from corn starch and sucrose and fibers are from cellulose.

[a] Determined within the diet by gas chromatography.

2.6. Lipid extraction and gas chromatography

Approximately 20 mg of frozen tissue was dissected from the frontal cortex of each mouse and used for fatty acid profile analyses. Weighed brain tissues were homogenized successively with 0.9%-NaCl, butylhydroxytoluene-methanol (Sigma-Aldrich) and chloroform (J.T. Baker, NJ, USA) using 22:3*n*-3 methyl ester as an internal standard (Nu-Chek Prep, MN, USA) at a concentration of 500 μg/g of tissue. After centrifugation at 2400 *g* for 7 min, the lower layer was collected. This procedure was repeated twice, and the two extracts were pooled and dried under a stream of N_2. Lipid extracts were transmethylated with methanol:benzene (4:1) and acetyl chloride at 98°C for 90 min. After cooling down, 6% K_2CO_3 was added. A 15-min centrifugation at 514 g allowed phase separation, and the upper layer was collected in a gas chromatography autosampler vial and capped under N_2. Fatty acid methyl esters were quantified using a model 6890 series gas chromatograph (Agilent Technologies, CA, USA) using a FAST-GC method. Five microliter of each sample were injected at a 25:1 split ratio. Tissue fatty acid methyl ester peak identification was performed by comparison to the peak retention times of a 28-component methyl ester reference standard (GLC-462; Nu-Chek Prep, MN, USA) [4].

2.7. Catecholamine quantification

Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were quantified by high-performance liquid chromatography (HPLC) using electrochemical detection [2]. Areas of the anterior striatum were dissected and homogenized with 200 μl perchloride acid (0.1 N). A volume of 50 μl of supernatant was directly injected into the chromatograph composed of a Waters 717 plus autosampler automatic injector, a Waters 1525 binary pump equipped with an Atlantis dC18 column, a Waters 2465 electrochemical detector and a glassy carbon electrode (Waters Limited, QC, Canada). Electrochemical potential was 10 nA. The mobile phase was composed of 8% MeOH, 0.47-M EDTA, 2-M NaCl, 0.69-M octanic sulfonic acid and 0.055-M NaH_2PO_4, and pH was adjusted at 2.9. The mobile phase was delivered at a flow rate of 1.0 ml/min. The identification of peaks was processed using the Breeze software (Waters). HPLC quantification was normalized with respect to protein concentration of each sample.

2.8. Striatal protein levels and Western blot analyses

Homogenization of samples was performed in eight volumes of lysis buffer (150-nM NaCl, 10-nM NaH_2PO_4, 1% v/v Triton X-100, 0.5% SDS and 0.5 sodium deoxycholate) with a mix of protease (Roche, ON, Canada) and phosphatase inhibitors (1-mM tetrasodium pyrophosphate and 50-mM sodium fluoride). The samples were sonicated (3×10-s pulse) and centrifuged for 20 min at 100,000 *g*, 4°C. The supernatant was collected and frozen at −80°C. The quantification of protein concentration was performed using the bicinchoninic acid protein assay kit (Thermo Fisher Scientifc). Twelve μg of proteins were heated with Laemmli loading buffer for 5 min and separated by electrophoresis on an 8% SDS-polyacrylamide gel. Proteins were then transferred onto 0.45 μm Immobilon PVDF membranes (Millipore, ON, Canada) and blocked with 5% skimmed milk and 1% BSA in PBS − 0.05% Tween for 1 h. The membranes were immunoblotted with the following primary antibodies: rabbit anti-tyrosine hydroxylase (TH, Pel-Freez, AR, USA, 1: 5000), rabbit anti-dopamine receptor D2 (Millipore; 1:1000), rabbit anti-nerve growth factor β (β-NGF, Millipore; 1:250), rabbit anti-tropomyosin receptor kinase B (TrkB, Millipore; 1:5,000, rabbit anti-neurotrophin-3 (NT3, Abcam Cambridge, UK; 1:750), mouse anti-postsynaptic density protein 95 (PSD95, Neuromab, CA, Neuromab; 1:5000), rabbit anti-growth-associated protein 43 (GAP43, Novus Biological, ON, Canada; 1: 5000), mouse anti-VGLUT1 (Neuromab, 1:4000), mouse anti-synaptophysin (Millipore; 1:10 000) and mouse anti-cytochrome oxidase 1 (CO1, Santa Cruz, TX, USA, 1:1000). After washing the membranes in PBS-Tween, they were incubated with the appropriate HRP-coupled anti-mouse or anti-rabbit secondary antibodies (Jackson Immunoresearch, PA, USA) followed by detection with chemiluniscence reagent (Luminata, Millipore). Chemiluminescence was measured with myECL Imager (Thermo Fisher Scientific Inc.). Immunoblot band intensity was quantified with Carestream Molecular Imaging Software (Molecular Imaging Software Version 4.0.5f7, Carestream Health, NY, USA).

2.9. Autoradiography

Striatal dopamine transporter (DAT) levels (Bregma level −0.46 mm) were evaluated using ^{125}I-RTI-121 [3β-(4-^{125}I-iodophenyl) tropane-2β-carboxylic acid isopropylester] (NEN-DuPont, MA, USA; 2200 Ci/mmol) according to previously published procedures [3,21]. Sections were preincubated at room temperature for 30 min in phosphate buffered saline (10.1-mM $NaHPO_4$, 1.8-mM KH_2PO_4, 137-mM NaCl and 2-mM KCl pH 7.4) followed by a 60-min incubation at room temperature with 20 pM ^{125}I-RTI-121. Ten nanomolar mazindol was used to evaluate non-specific binding (Novartis, Basel, Switzerland). After two washes of 20 min in PBS, sections were briefly (10 s) rinsed in distilled water (all at 4°C). Finally, the slide-mounted tissue sections were dried overnight at room temperature and exposed on ^{3}H-sensitive Kodak Biomax film (Sigma–Aldrich) for 24 h.

2.10. Immunohistochemical evaluation of the striatal and nigral DAergic system

Brain sections of the substantia nigra pars compacta (SNpc) and striatum were respectively post-fixed in PFA 4% pH 7.4 for 48 h, followed by a 20% sucrose solution or post-fixed 1 h in 4% PFA. Brains were sliced in coronal sections of 20 (SNpc) or 12 (striatum)-μm in thickness using a microtome or a cryostat, respectively. Brain sections were washed in 0.1M PBS and subsequently placed in 3% hydrogen peroxide for 30 min at room temperature. Sections were washed in PBS and placed in a blocking solution (PBS, 0.1% Triton X-100 (Sigma–Aldrich) containing 5% normal goat serum (NGS; Wisent, QC, Canada) for 30 min. The sections were then incubated overnight at 4°C with the primary antibody rabbit anti-TH (PEL-Freez, 1/5000 for SNpc and 1/500 for striatum) in the blocking solution. Slices were washed three times in PBS, incubated with a secondary biotinylated goat anti-rabbit IgG (Jackson Immunoresearch) followed by an incubation with an avidin-biotin peroxidase complex (ABC; Vector Laboratories, CA, USA) for 1 h at room temperature. The antibody binding was detected by placing the sections in a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich) and 0.1% of 30% hydrogen peroxide in PBS. The reaction was stopped by extensive washes in PBS. Following the DAB staining, SNpc sections were counterstained with cresyl violet (Sigma–Aldrich), dehydrated and coverslipped.

2.11. In situ hybridization

The dopamine transporter (DAT) probe, a 2238-bp fragment, was cloned into pBluescript II SK plasmid. Linearization was made with the *Not*I enzyme. The antisense probe was synthesized with 35S-UTP and T7 RNA polymerase, as described previously [2]. Brain sections mounted onto superfrost slides were post-fixed 20 min in 4% PFA and rinsed twice with PBS for 5 min. They were then submerged in a protease K solution at 37°C, washed for a few seconds in DEPC water and incubated in an acetylation bath with triethanolamine (100-mM TEA, pH 8.0) and anhydride acetic acid solution for 10 min. Slides were rinsed twice in a standard salt sodium citrate (SSC) solution, dehydrated and air dried at room temperature. The ^{35}S-UTP-radiolabeled complementary RNA (cRNA) probe was added to the hybridization mix (50% dextran sulfate and 50% deionized formamide in Denhart's solution) and heated at 80°C for 5 min. Each slide was covered with 100 μl of the hybridization solution and coverslipped. The hybridization took place on a slide warmer at 58°C overnight. After hybridization, slides were placed in 4°C SSC for 30 min to remove coverslips and washed four times in SSC baths. Slides were incubated at 37°C for 30 min in an RNase A solution (0.2% RNase A; 10% 10× RNase A buffer) followed by four successive SSC baths. Finally, tissues were dehydrated and exposed on Kodak Biomax films for 24 h.

Following film exposure, slides were defatted with four sequential 5-min baths of ethanol, two baths of Citrisolv (Thermo Fisher Scientific Inc.) (10 min and 30 min, respectively) and three 5-min baths of ethanol (100%). They were then dipped in NTB emulsion (Kodak) and incubated at 42°C, air dried for 2 h and incubated in the dark for 7 days at 4°C. The emulsion was then developed in D-19 developer (3.5 min; Kodak), rinsed in deionized water and fixed (5 min) in Rapid Fixer solution (Kodak). Slides were rinsed in deionized water for 1 h and then stained with 0.25% thionine (Sigma–Aldrich) (30 s), followed by water, ethanol dips and an additional three ethanol (1 min) and three Citrisolv baths (3 min each). Finally, slides were coverslipped with mounting media (DPX) (Sigma–Aldrich).

2.12. Quantification of nigral TH-immunoreactive neurons and DAT mRNA expressing cells

The number of nigral TH+ and DAT mRNA+ neurons was determined by stereological counts under brightfield illumination (Bousquet, 2008). Seven sections at 125 μm (for TH) and 250-μm intervals (for DAT) through the SNpc (AP: levels of −2.70 mm to −3.80 mm [19]) were analyzed using the Stereo investigator software (MicroBrightfield) integrated to a E800 Nikon microscope (Nikon Canada Inc., ON, Canada) [22,23]. Unbiased cell counts were further performed blindly by two independent investigators. Analyses of immunoreactive profiles were restricted to the SNpc and thus excluded the ventral tegmental area.

The dendritic arborization of nigral TH+ neurons of 6-OHDA lesioned mice, visible under brightfield, was drawn using Neurolucida (MBF bioscience, VT, USA) and analyzed using Neuroexplorer (Nex Technologies, AL, USA). This was performed on the lesioned side using five animals per group. Analyses included the total length of neurites (computed by adding the lengths of each segment, referred to as B1-branch 1, B2-branch 2 and B3-branch 3), the percentage of neurite presenting 0, 1 or 2 branches and the total neurite surface, as well as the perimeter and area of cell bodies as previously described [18].

2.13. Statistical analyses

Results for each group are presented as the mean±S.E.M. The normality of the distribution was determined with Agostino & Pearson's omnibus normality test. In cases of comparable group variances (Bartlett's test) and normal distribution, statistical differences were determined using one-way analysis of variance (ANOVA) followed by post-hoc (Tukey's) tests for comparisons between groups. For comparisons between two groups, a Student's *t* test (normal distribution) or Mann–Whitney test was performed. Two-way ANOVA were used to compare two categorical variables. Note that outliers were removed from final analysis. These were identified using the ROUT method (0.5%), which is based on the analyses of nonlinear regression and evaluation of the residuals of the robust fit. All statistical analyses were performed using the JMP (Version 11; SAS Institute Inc., Cary, IL, USA) and Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) software. For all statistical analyses, alpha was set to 0.05.

3. Results

3.1. Enriched n-3 PUFA intake does not improve motor impairments in 6-OHDA lesioned mice

We first assessed whether n-3 PUFA intake improved the motor behavior of 6-OHDA-lesioned mice (Fig. 1). As expected, the 6-OHDA lesion induced an increase in apomorphine-induced rotations, as revealed by both one-way and two-way ANOVAs (Fig. 1B). However, the n-3 PUFA-enriched diet did not improve any of the behavioral outcomes measured, which included apomorphine-induced rotations (Fig. 1B), asymmetry in use of the front paws in the cylinder test (Fig. 1C), adjusting steps (Fig. 1D) and locomotor activity measured as the total distance travelled in the openfield (Fig. 1E). All behavioral measures remained unchanged across all experimental groups.

3.2. Enriched n-3 PUFA intake translates into measurable accumulation of fatty acids in cortical brain structures

As previously demonstrated [2], a high dietary intake of n-3 PUFAs over a period of 6 weeks (from 3 to 9 weeks post-lesion) increased levels of n-3 PUFAs as well as DHA concentrations in the motor cortex of animals on such diets (Fig. 2A, C). In contrast, the levels of total n-6 PUFA and arachidonic acid were decreased following n-3 PUFA intake (Fig. 2B, D). The overall n-3 PUFA:n-6 PUFA ratio was further increased following the assigned n-3 PUFA supplemented diet (Fig. 2E). It should be noted that the 6-OHDA lesion had no effect on cortical fatty acid profiles.

3.3. Beneficial effects of enriched n-3 PUFA intake on several striatal components of the DAergic system

As evaluated by HPLC, the 6-OHDA lesion induced a significant reduction of DA concentrations in the lesioned striatum. Interestingly, n-3 PUFA intake, in a neurorescue/neurorestorative context (3 weeks post-lesion) led to 111% higher striatal DA concentrations compared to animals on the control diet, with a metabolic ratio of DA (DOPAC+HVA/DA) that was also increased in the lesioned mice fed with n-3 PUFA dietary (Fig. 3A, B). Similarly, TH levels measured by immunoblotting were 89% higher in animals fed a rich diet in omega-3 compared to animals fed the control diet (Fig. 3C). However, protein content for the dopamine receptor type 2 (D2) was not significantly modulated by either the lesion or the diet (Fig. 3D).

The 6-OHDA lesion induced a significant loss of both striatal TH+ fibers quantified by IHC (Fig. 3E) and ^{125}I-RTI-121-specific binding to DAT (Fig. 3F) and, as confirmed, by two-way ANOVA. However, one-way ANOVA revealed that the 6-OHDA lesion-induced decreases in both TH fibers, and DAT levels were significant only in animals on the control diet ($P<0.0001$ and $P=0.001$, respectively) and not in animals on the omega-3 diet ($P=0.067$ and $P=0.121$, respectively), when compared to their respective controls (Fig. 3E, F). In other words, the depleting effect of the 6-OHDA lesion on TH terminals and DAT was not statistically significant in mice fed with the n-3 PUFA diet, as measured 9-weeks post-lesion, suggesting that the n-3 PUFA-enriched food supplementation led to a partial recovery of these two markers of DAergic terminals.

3.4. Enriched n-3 PUFA intake does not impact levels of striatal neurotrophic factors, synaptic proteins or metabolic markers

To probe for mechanistic correlates, protein levels for the neurotrophic factors NGF, TrkB (BDNF receptor) and NT3 were measured by

western blot, but no changes were observed (Fig. 4A, B, C). Similarly, no significant differences were detected between the striatal synaptic proteins PSD95, GAP43, VGLUT1 and synaptophysin between groups (Fig. 4D, E, F, G). Finally, levels of the metabolic marker CO1 also remained similar across groups (Fig. 4H).

3.5. Effects of enriched n-3 PUFA intake on the nigral DAergic system: augmentation of cell body size

As expected, 6-OHDA lesions induced a decrease in the number of DAergic cells in the SNpc, as quantified by stereological counts of TH+ cells (Fig. 5A). However, no significant difference was observed between animals fed with the n-3 PUFA enriched or with the control diet (Fig. 5A). Similarly, the number of DAT-mRNA expressing cells was decreased by the lesion but was not rescued/restored by n-3 PUFA intake (Fig. 5B).

To evaluate more subtle effects of n-3 PUFA on the nigral DAergic system, we measured the size of TH+ neurons in the SNpc. Interestingly, DAergic cell bodies measured in the 6-OHDA-lesioned mice were greater in size (both perimeter and areas covered) with the enriched diet. The n-3 PUFA supplementation increased the perimeter of DAergic neurons (+7%, $P=0.0173$) and enhanced neuronal cell body area by 21% ($P=0.0199$) (Fig. 5C). Correlations were established between DAergic cell areas and perimeters with the brain n-3: n-6 PUFA ratio ($r^2=0.3266$, $P=0.1802$; $r^2=0.6381$, $P=0.0312$, respectively). On the other hand, changes in length (46.19±2.37 μm ctrl, 50.72±4.39 μm n-3 PUFA), surface (100.85±8.25 μm ctrl, 115.63± 12.39 μm n-3 PUFA) and volume (24.42±3.7 μm ctrl, 27.21±4.28 μm n-3 PUFA) of DAergic neurites did not reach statistical significance when comparing n-3-PUFA-treated and untreated mice.

4. Discussion

Having previously established that n-3 PUFAs could have a neuroprotective effect in animal models of PD [2], we investigated their neurorescue/neurorestorative potential on the nigrostriatal DAergic system, 3 weeks following a 6-OHDA lesion, when DAergic degeneration has been initiated and is already significant [18]. Our data show that exposing the denervated nigrostriatal DAergic system to a DHA-enriched environment promotes the recovery of DAergic terminals, as testified by enhanced striatal DA levels, TH immunostaining and DAT-binding sites. Although cell counts of TH+ nigral neurons did not differ between lesioned and n-3 PUFA conditions, SNpc DAergic neurons of DHA-treated animals displayed larger cell bodies, suggesting enhanced cellular activity [24]. Overall, our findings support the impact of DHA supplementation in strengthening the DAergic system.

More than 50 years after its discovery, the loss of striatal DA remains the pathognomonic feature of PD, closely associated to the main motor symptoms. The dietary-induced increase of striatal DA concentrations observed here suggests that brain DHA favored the accumulation of DA in presynaptic vesicles and/or release in this brain structure. Although not associated with measurable behavioral changes, such a stimulating effect of DHA is consistent with microdialysis studies showing an increased release of DA in the striatum and in several cortical regions following intake of n-3 PUFA [25,26]. In a paradigm where the omega-3-enriched diet was administrated 2 months prior to the 6-OHDA lesion, however, such striatal DA-related changes have not been observed [27]. This experimental design, which calls for a neuroprotective approach and in which animals were sacrificed almost immediately after the lesion

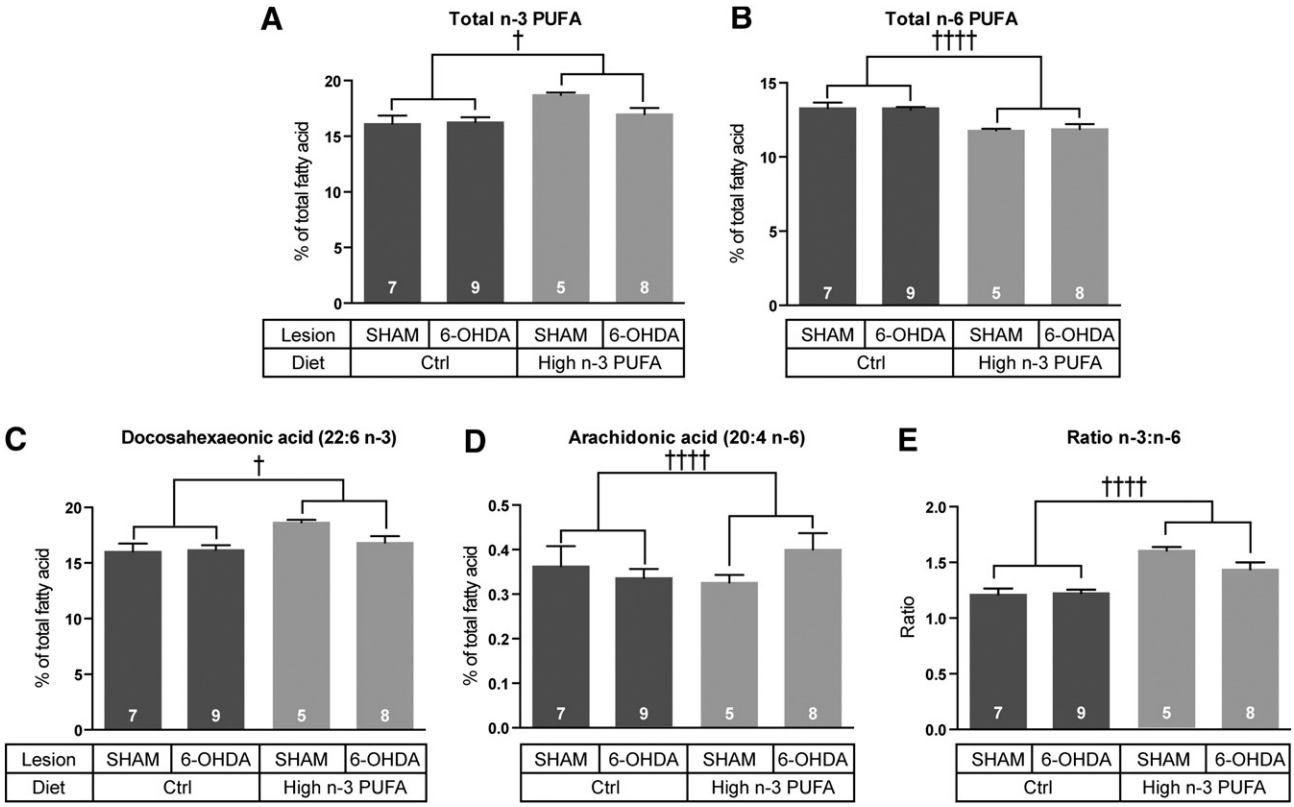

Fig. 2. **Brain levels of n-3 PUFAs.** Supplementation with n-3 PUFAs led to a higher concentration of total n-3 PUFA (A), a lower concentration of n-6 PUFA (B), higher DHA (22:6) levels (C), lower arachidonic (20:4) (D) acid levels as well as an overall higher n-3 PUFA: n-6 PUFA ratio (E), as measured in the cortex. The number written in each column represents the number of animals. Statistical analyses were performed using two-way ANOVA † $P<0.05$, †††† $P<0.0001$. Values are expressed as means±S.E.M. Abbreviations: 6-OHDA: 6-hydroxydopamine; Ctrl: control; n-3: omega-3; n-6: omega-6; PUFA: polyunsaturated fatty acid.

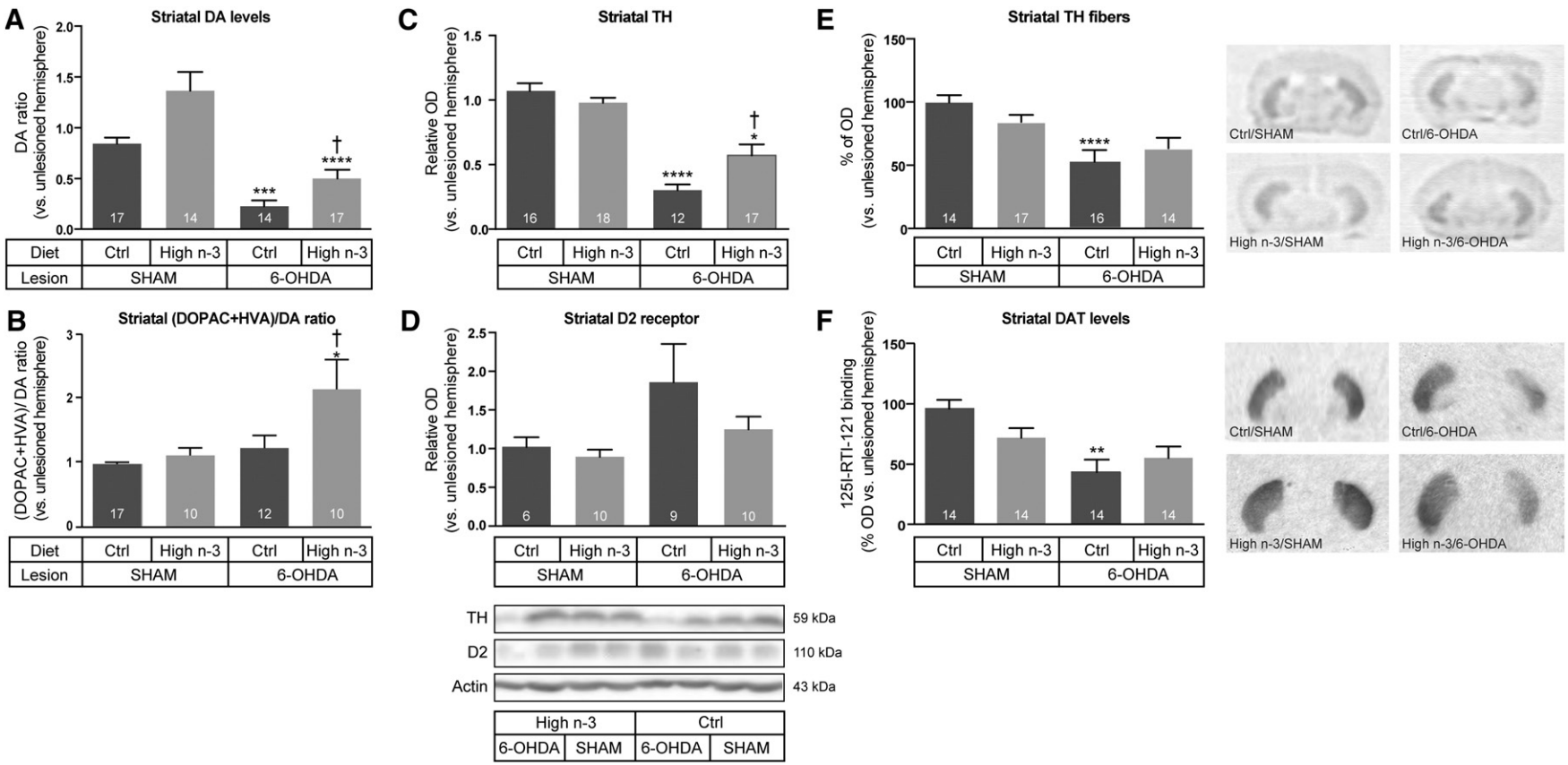

Fig. 3. **Dietary n-3 PUFA effects on striatal DAergic components.** Enriched dietary intake in n-3 PUFAs increased striatal dopamine (DA) concentrations (A) as well as DA metabolites (DOPAC+HVA) and DA ratio (DOPAC+HVA/DA) (B), as measured by HPLC. Enriched dietary intake in n-3 PUFAs further increased striatal TH fiber density (C), blunted the 6-OHDA-induced decrease in striatal TH+ fibers (E) and ^{125}I-RTI-121 specific binding to DAT (F), while D2 receptor levels were unchanged (D). Representative images of western blots (C–D), TH immunostaining (E) and ^{125}I-RTI-121 autoradiography (F). Western blot bands were cropped from the same membrane. The number written in each column represents the number of animals. Statistical analyses were performed using Student *t* test and Mann–Whitney test (only for C). † $P<0.05$ lesioned 6-OHDA fed with n-3 PUFA diet versus ctrl diet; ** $P<0.01$, ***$P<0.001$, **** $P<0.0001$ compared to unlesioned groups fed with the identical diets. Values are expressed as means±S.E.M. Abbreviations: 6-OHDA: 6-hydroxydopamine; Ctrl: control; D2: dopamine receptor type 2, DA: dopamine; DAT: dopamine transporter; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; n-3: omega-3. OD: optical density; TH: tyrosine hydroxylase.

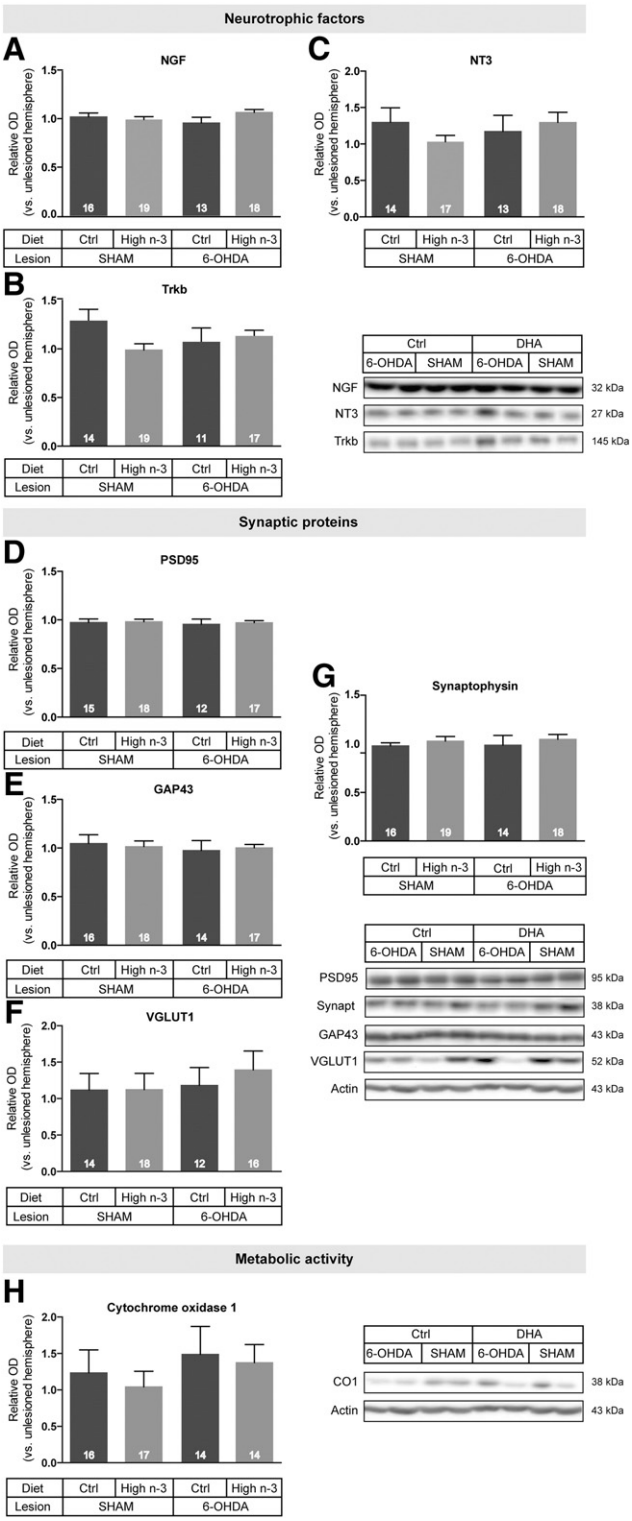

Fig. 4. **Dietary n-3 PUFA effects on the striatal DAergic system are not associated with changes in neurotrophic factors, synaptic proteins or metabolic markers.** Levels of striatal neurotrophic factors NGF (A), trkB (B), NT3 (C), synaptic proteins PSD95 (D), GAP43 (E), VGLUT1 (F), synaptophysin (G) or metabolic activity CO1 (H) were not modulated by an enriched n-3 PUFA diet. Western blot bands were cropped from the same membrane. The number written in each column represents the number of animals. Statistical analyses were performed using Student *t* test. Values are expressed as means±S.E.M. Abbreviations: 6-OHDA: 6-hydroxydopamine; CO1; cytochrome oxidase 1. Ctrl: control; GAP43: neuronal growth-association protein; n-3: omega-3; NGF: neurotrophic growth factor; NT3: neurotrophin-3; OD: optical density; PSD95: postsynaptic density protein 95; Synapt: synaptophysin; TH: tyrosine hydroxylase; TrkB: brain derived neurotrophic factor tropomysosin receptor kinase B; VLGUT1: vesicular glutamate transporters.

(5-day post-lesion), may account for this discrepancy. The heightened striatal TH immunoreactivity, as well as the absence of statistical differences between TH fibers and DAT expression in the DHA-treated animals post-lesion observed in our study, converges to suggest that DHA stimulates the recovery of striatal TH terminals subsequently to the degeneration of SNpc neurons.

The vast majority of pre-clinical studies testing potential interventions in PD models have focused on pre-treatment — before the neurotoxic injury — thereby probing for neuroprotective effects of the compound under investigation [2,28,29], and this is the case for n-3 PUFAs as well [2,3]. Epidemiological evidence is consistent with a possible preventive effect of DHA [30]. In contrast, very few compounds have been shown to potentiate an already lesioned DAergic system, despite the fact that this is what virtually all PD patients need. Here, we thus opted for a neurorestorative experimental design, where DHA supplementation was administered following the initiation of DAergic denervation. There are no perfect models of PD, particularly to study neurorestoration [28,31]. Whereas MPTP administration is the most commonly used paradigm to investigate neuroprotection, it is usually associated with a significant recovery in the weeks following the lesion [32–35], which limits its use to study neurorestoration in mice. In contrast, stereotaxic administration of 6-OHDA triggers a replicable and extensive DAergic denervation (>70%) [36] which allows the detection of post-lesion recovery *in vivo* with higher sensitivity and accuracy. The 6-OHDA model is also well suited for the assessment of a behavioral phenotypes [20,28,31]. In addition, an attractive characteristic of the unilateral 6-OHDA models is that the unlesioned side of each animal can be used as its own control, lowering the impact of mouse-to-mouse variation.

In our chosen paradigm, it remains difficult to attribute the DA recovery observed to either a neurorestorative process or to a recovery of the DAergic system driven by enhanced activity and/or dendritic sprouting of remaining cells. Neurorestoration is usually defined as replacing dying or degenerated neurons with viable cells [37,38]. Without an actual increase in the number of TH+ nigral DAergic cell bodies *per se*, and especially in the context of post-lesion therapy, it is unclear whether we can conclude on neurorestorative properties. It is possible that an intervention longer than the one used here (6 weeks) may have had more sustained effects on brain fatty acid profiles and even led to neurogenesis. In contrast, the neuroprotective impact of n-3 PUFA was more prominent on nigral cells than on DAergic terminals [2], which may imply different mechanisms.

On the other hand, the observed n-3 PUFA-induced rise in striatal DA could also be explained by post-lesion compensatory mechanisms, predominant at the DAergic terminals. The importance of this should not be underestimated in PD. The disease symptomatology is ultimately the result of not only nigral denervation, but also the failure of compensatory mechanisms [39]. Motor symptoms occur only when 50 to 80% of DAergic cells have degenerated, as documented in both PD patients and animal models [40–43]. Several lines of evidence for substantial post-lesion recovery of striatal DA have been reported in rodent and primate PD models [44,45]. Thus, despite the total number of DAergic neurons remaining the same, motor function can be maintained by compensatory mechanisms for years before frank motor features are detected. Therefore, a DAergic functional recovery focalized in the striatum stands as an interesting explanation for the observed effects of DHA. Such neurorecovery of DAergic terminals, if sustained overtime, may ultimately be as clinically relevant/significant as neurorestoration.

Possible compensatory mechanisms underlying the effects of n-3 PUFA on striatal dopamine levels include (a) a decrease in metabolic degradation of DA; (b) activation of glutamate-related compensatory mechanisms; and/or (c) an increased release of DA from striatal terminals originating from either the SN or the ventral tegmental area. The first possibility is unlikely since an increase in (HVA+DOPAC)/DA

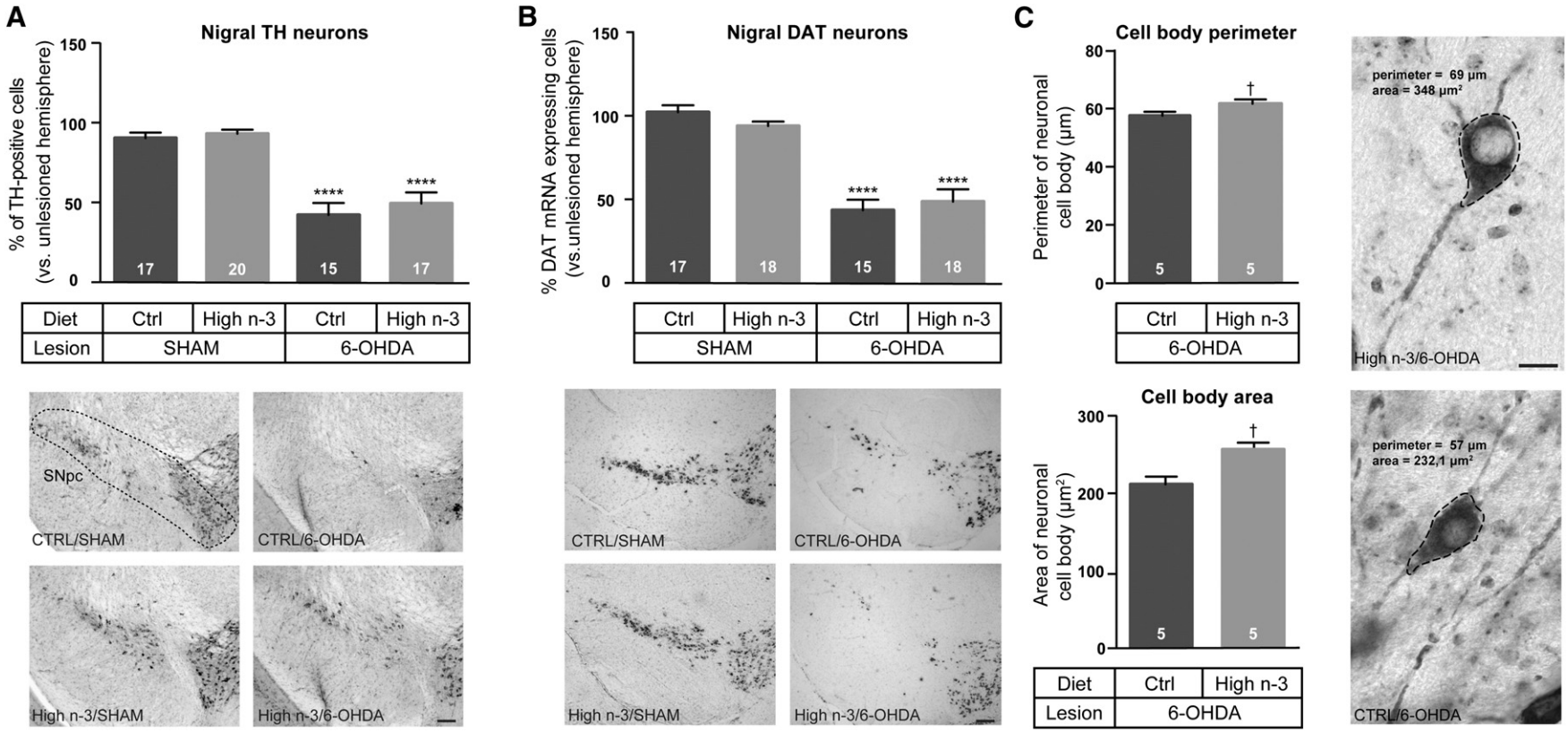

Fig. 5. **Dietary n-3 PUFA effects on the nigral DAergic system.** (A–B) n-3 PUFA intake did not reverse the 6-OHDA-induced decrease in the number of TH (A) nor DAT (B) positive cells of the SNpc. Representative photomicrographs illustrating TH and DAT immunostained cells in the SNpc for each condition. In contrast, n-3 PUFAs led to a significant increase in the perimeter and total area covered by the cell body of nigral TH+ neurons (C). Representative images of nigral TH+ cell bodies. The number written in each column represents the number of animals. Statistical analyses were performed using Student *t* test, † $P<0.05$ lesioned 6-OHDA fed with n-3 PUFA dietary versus ctrl diet; **** $P<0.0001$ compare to the unlesioned group fed with the same diet. Values are expressed as means±S.E.M. Scale bars=A,B: 100 µm; C: 20 µm. Abbreviations: 6-OHDA: 6-hydroxydopamine; Ctrl: control; DAT: dopamine transporter; n-3 omega-3; TH: tyrosine hydroxylase.

ratio was detected in animals exposed to n-3 PUFAs, which is rather consistent with an enhanced axonal release of DA, and thereby faster metabolism in the synaptic cleft. Concerning the second hypothesis, evidence suggest that changes in cortico-cortical glutamate input plays a role in compensating for nigral denervation, although the mechanisms for this remain unclear [46–49]. However, our results show no difference in striatal CO1 or VGLUT1, which does not suggest massive changes in mitochondrial activity nor striatal glutamatergic transmission following n-3 PUFA treatment. On the other hand, our data provide strong evidence to support the third hypothesis. Exposing the lesioned striatum to DHA led to increased TH levels and blunted the 6-OHDA-induced decreases in DAT or TH terminals, which is consistent with an enhanced arborescence of striatal DAergic terminals. Our morphology analysis showed that SN DAergic cell bodies are enlarged, while DAergic neuronal counts remained the same, suggesting increased cellular activity rather than cell number. Consistently, recent studies report a reduction of cell body size following n-3 PUFA deprivation [15,50,51]. Moreover, neuronal membrane expansion [52] and neurite outgrowth [53] are documented effects of n-3 PUFA *in vivo*. It is also in agreement with the previously reported compensatory sprouting of DA fibers, originating mostly from spared ventral tegmental DAergic neurons in denervated animals [54]. The timing of the diet with respect to the lesion is extremely important as the effects of DHA may be greater or much more noticeable at the nigral level if the diet is indeed administered more rapidly after the 6-OHDA lesion, when dying neurons can still be rescued. The intrastriatal injection of 6-OHDA creates a progressive retrograde degeneration that takes place over several days/weeks [55]. Sauer and Oertel demonstrated that the lower point of denervation is reached 3 weeks post-lesion, when it also seems to stabilize. Here, we aimed to intervene after the acute neurotoxic effect of 6-OHDA, to have a greater chance to detect true neurorestoration and to better reflect potential interventions in clinical PD. It is also very likely that a diet administration exceeding 6 weeks would have had a cumulative or greater effect of the DAergic system. Future studies assessing the extent of DA-recovery in relation to various degrees of degeneration as well as periods of DHA administration are required to clarify these questions. Overall, our observations support the hypothesis that DHA supplementation results in surviving DAergic cells having expanded striatal axonal terminals and producing/secreting DA, thereby compensating for the death of neighboring cells; a phenomenon we have recently shown with other drugs tested in an identical context [18].

The vast majority of clinical trials in the field of PD have focused on symptomatic effects, mostly because identifying disease-modifying effects requires large trials with complex design. Although large-scale preventive interventions are worth considering, a post-injury paradigm is more compatible with a clinical use of n-3 PUFA supplementation in human PD, which would occur after patients consult their neurologist and receive their diagnosis, at a point in time when neurodegeneration processes have long begun [56]. Meanwhile, pre-clinical assays provide a key advantage because they are readily suitable to investigate restoration over time. Using a paradigm in which DHA treatment was implemented upon a lesioned DAergic system, we demonstrate that DHA intake leads to higher DA concentrations in the striatum along with an increase in DAergic terminals. Although cell count remained unchanged, enlarged DAergic cells following DHA treatment further provide evidence that compensatory mechanisms are at play. If such compensatory mechanisms leading to a degree of functional recovery could be achieved by natural compounds, such as n-3 PUFAs, the benefits to patients could be considerable.

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References

[1] de Lau LML, Breteler MMB. Epidemiology of Parkinson's disease. Lancet Neurol 2006;5:525–35. http://dx.doi.org/10.1016/S1474-4422(06)70471-9.
[2] Bousquet M, Saint-Pierre M, Julien C, Salem N, Cicchetti F, Calon F. Beneficial effects of dietary omega-3 polyunsaturated fatty acid on toxin-induced neuronal degeneration in an animal model of Parkinson's disease. FASEB J 2008;22:1213–25. http://dx.doi.org/10.1096/fj.07-9677com.
[3] Bousquet M, Calon F, Cicchetti F. Impact of omega-3 fatty acids in Parkinson's disease. Ageing Res Rev 2011;10:453–63. http://dx.doi.org/10.1016/j.arr.2011.03.001.
[4] Bousquet M, Gue K, Emond V, Julien P, Kang JX, Cicchetti F, et al. Transgenic conversion of omega-6 into omega-3 fatty acids in a mouse model of Parkinson's disease. J Lipid Res 2011;52:263–71. http://dx.doi.org/10.1194/jlr.M011692.
[5] Olanow CW, Schapira AHV. Therapeutic prospects for Parkinson disease. Ann Neurol 2013;74:337–47. http://dx.doi.org/10.1002/ana.24011.
[6] Meissner WG, Frasier M, Gasser T, Goetz CG, Lozano A, Piccini P, et al. Priorities in Parkinson's disease research. Nat Rev Drug Discov 2011;10:377–93. http://dx.doi.org/10.1038/nrd3430.
[7] Julien C, Berthiaume L, Hadj-Tahar A, Rajput AH, Bédard PJ, Paolo TD, et al. Postmortem brain fatty acid profile of levodopa-treated Parkinson disease patients and parkinsonian monkeys. Neurochem Int 2006;48:404–14. http://dx.doi.org/10.1016/j.neuint.2005.12.002.
[8] Salem N, Litman B, Kim HY, Gawrisch K. Mechanisms of action of docosahexaenoic acid in the nervous system. Lipids 2001;36:945–59. http://dx.doi.org/10.1007/s11745-001-0805-6.
[9] Calon F, Lim GP, Yang F, Morihara T, Teter B, Ubeda O, et al. Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. Neuron 2004;43:633–45. http://dx.doi.org/10.1016/j.neuron.2004.08.013.
[10] Bazan NG. The docosanoid neuroprotectin D1 induces homeostatic regulation of neuroinflammation and cell survival. Prostaglandins Leukot Essent Fat Acids 2013;88:127–9. http://dx.doi.org/10.1016/j.plefa.2012.08.008.
[11] Paterniti I, Impellizzeri D, Di Paola R, Esposito E, Gladman S, Yip P, et al. Docosahexaenoic acid attenuates the early inflammatory response following spinal cord injury in mice: in-vivo and in-vitro studies. J Neuroinflammation 2014;11:6. http://dx.doi.org/10.1186/1742-2094-11-6.
[12] Lalancette-Hébert M, Julien C, Cordeau P, Bohacek I, Weng Y-C, Calon F, et al. Accumulation of dietary docosahexaenoic acid in the brain attenuates acute immune response and development of postischemic neuronal damage. Stroke 2011;42:2903–9. http://dx.doi.org/10.1161/STROKEAHA.111.620856.
[13] Akbar M, Calderon F, Wen Z, Kim H-Y. Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. Proc Natl Acad Sci U S A 2005;102:10858–63. http://dx.doi.org/10.1073/pnas.0502903102.
[14] Arsenault D, Julien C, Tremblay C, Calon F. DHA improves cognition and prevents dysfunction of entorhinal cortex neurons in 3xTg-AD mice. PLoS ONE 2011;6:e17397. http://dx.doi.org/10.1371/journal.pone.0017397.
[15] Ahmad A, Murthy M, Moriguchi T, Salem N, Greiner RS. A decrease in cell size accompanies a loss of docosahexaenoate in the rat hippocampus. Nutr Neurosci 2002;5:103–13. http://dx.doi.org/10.1080/10284150290018973.
[16] Bousquet M, Gibrat C, Saint-Pierre M, Julien C, Calon F, Cicchetti F. Modulation of brain-derived neurotrophic factor as a potential neuroprotective mechanism of action of omega-3 fatty acids in a parkinsonian animal model, Prog Neuropsychopharmacol Biol Psychiatry. 2009;33:1401–8. http://dx.doi.org/10.1016/j.pnpbp.2009.07.018.
[17] Cardoso HD, Santos Junior dos EF, de Santana DF, Gonçalves-Pimentel C, Angelim MK, Isaac AR, et al. Omega-3 deficiency and neurodegeneration in the substantia nigra: involvement of increased nitric oxide production and reduced BDNF expression, Biochim Biophys Acta. 2010;1840:1–11. http://dx.doi.org/10.1016/j.bbagen.2013.12.023.
[18] Cisbani G, Drouin-Ouellet J, Gibrat C, Saint-Pierre M, Lagacé M, Badrinarayanan S, et al. Cystamine/cysteamine rescues the dopaminergic system and shows neurorestorative properties in an animal model of Parkinson's disease. Neurobiol Dis 2015;82:430–44. http://dx.doi.org/10.1016/j.nbd.2015.07.012.
[19] Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. Academic Press; 2008.
[20] Glajch KE, Fleming SM, Surmeier DJ, Osten P. Sensorimotor assessment of the unilateral 6-hydroxydopamine mouse model of Parkinson's disease. Behav Brain Res 2012;230:309–16. http://dx.doi.org/10.1016/j.bbr.2011.12.007.
[21] Calon F, Lavertu N, Lemieux AM, Morissette M, Goulet M, Grondin R, et al. Effect of MPTP-induced denervation on basal ganglia GABA(B) receptors: correlation with

dopamine concentrations and dopamine transporter. Synapse 2001;40:225–34. http://dx.doi.org/10.1002/syn.1045.
[22] Drouin-Ouellet J, Gibrat C, Bousquet M, Calon F, Kriz J, Cicchetti F. The role of the MYD88-dependent pathway in MPTP-induced brain dopaminergic degeneration. J Neuroinflammation 2011;8:137. http://dx.doi.org/10.1186/1742-2094-8-137.
[23] Gibrat C, Saint-Pierre M, Bousquet M, Lévesque D, Rouillard C, Cicchetti F. Differences between subacute and chronic MPTP mice models: investigation of dopaminergic neuronal degeneration and α-synuclein inclusions. J Neurochem 2009;109:1469–82. http://dx.doi.org/10.1111/j.1471-4159.2009.06072.x.
[24] Arsenault D, Julien C, Calon F. Chronic dietary intake of α-linolenic acid does not replicate the effects of DHA on passive properties of entorhinal cortex neurons. Br J Nutr 2011;107:1099–111. http://dx.doi.org/10.1017/S0007114511004089.
[25] Chalon S. Omega-3 fatty acids and monoamine neurotransmission. Prostaglandins Leukot Essent Fat Acids 2006;75:259–69. http://dx.doi.org/10.1016/j.plefa.2006.07.005.
[26] Shin SS, Dixon CE. Oral fish oil restores striatal dopamine release after traumatic brain injury. Neurosci Lett 2011;496:168–71. http://dx.doi.org/10.1016/j.neulet.2011.04.009.
[27] Delattre AM, Kiss ÃG, Szawka RE, Anselmo-Franci JA, Bagatini PB, Leal Xavier der LÃ, et al. Evaluation of chronic omega-3 fatty acids supplementation on behavioral and neurochemical alterations in 6-hydroxydopamine-lesion model of Parkinson's disease. Neurosci Res 2010;66:256–64. http://dx.doi.org/10.1016/j.neures.2009.11.006.
[28] Bezard E, Przedborski S. A tale on animal models of Parkinson's disease. Mov Disord 2011;26:993–1002. http://dx.doi.org/10.1002/mds.23696.
[29] Bezard E. A call for clinically driven experimental design in assessing neuroprotection in experimental Parkinsonism. Behav Pharmacol 2006;17:379–82 [PMID 16940758].
[30] de Lau LML, Bornebroek M, Witteman JCM, Hofman A, Koudstaal PJ, Breteler MMB. Dietary fatty acids and the risk of Parkinson disease: the Rotterdam study. Neurology 2005;64:2040–5. http://dx.doi.org/10.1212/01.WNL.0000166038.67153.9F.
[31] Bezard E, Yue Z, Kirik D, Spillantini MG. Animal models of Parkinson's disease: limits and relevance to neuroprotection studies. Mov Disord 2012;28:61–70. http://dx.doi.org/10.1002/mds.25108.
[32] Mitsumoto Y, Watanabe A, Mori A, Koga N. Spontaneous regeneration of nigrostriatal dopaminergic neurons in MPTP-treated C57BL/6 mice. Biochem Biophys Res Commun 1998;248:660–3. http://dx.doi.org/10.1006/bbrc.1998.8986.
[33] L'Episcopo F, Tirolo C, Testa N, Caniglia S, Morale MC, Deleidi M, et al. Plasticity of subventricular zone neuroprogenitors in MPTP (1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine) mouse model of Parkinson's disease involves cross talk between inflammatory and Wnt/-Catenin signaling pathways: functional consequences for neuroprotection and repair. J Neurosci 2012;32:2062–85. http://dx.doi.org/10.1523/JNEUROSCI.5259-11.2012.
[34] Date I, Felten DL, Felten SY. Long-term effect of MPTP in the mouse brain in relation to aging: neurochemical and immunocytochemical analysis. Brain Res 1990;519:266–76. http://dx.doi.org/10.1016/0006-8993(90)90088-S.
[35] Schneider JS, Rothblat DS. Neurochemical evaluation of the striatum in symptomatic and recovered MPTP-treated cats. Neuroscience 1991;44:421–9. http://dx.doi.org/10.1016/0306-4522(91)90066-W.
[36] Jackson-Lewis V, Blesa J, Przedborski S. Animal models of Parkinson's disease. Parkinsonism Relat Disord 2012;18(Suppl. 1):S183–5. http://dx.doi.org/10.1016/S1353-8020(11)70057-8.
[37] Mandel S, Grünblatt E, Riederer P, Gerlach M, Levites Y, Youdim MBH. Neuroprotective strategies in Parkinson's disease. CNS Drugs 2003;17:729–62. http://dx.doi.org/10.2165/00023210-200317100-00004.
[38] Airavaara M, Voutilainen MH, Wang Y, Hoffer B. Neurorestoration. Parkinsonism Relat Disord 2012;18:S143–6. http://dx.doi.org/10.1016/S1353-8020(11)70045-1.
[39] Bezard E, Gross CE, Brotchie JM. Presymptomatic compensation in Parkinson's disease is not dopamine-mediated. Trends Neurosci 2003;26:215–21. http://dx.doi.org/10.1016/S0166-2236(03)00038-9.
[40] Elsworth JD, Deutch AY, Redmond DE, Sladek JR, Roth RH. MPTP-induced parkinsonism: relative changes in dopamine concentration in subregions of substantia nigra, ventral tegmental area and retrorubral field of symptomatic and asymptomatic vervet monkeys. Brain Res 1990;513:320–4. http://dx.doi.org/10.1016/0006-8993(90)90474-P.
[41] Bezard E, Dovero S, Prunier C, Ravenscroft P, Chalon S, Guilloteau D, et al. Relationship between the appearance of symptoms and the level of nigrostriatal degeneration in a progressive 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned macaque model of Parkinson's disease. J Neurosci 2001;21:6853–61 [PMID 11517273].
[42] Stoessl AJ. Neuroimaging in Parkinson's disease. Neurotherapeutics 2011;8:72–81. http://dx.doi.org/10.1007/s13311-010-0007-z.
[43] Berti V, Pupi A, Mosconi L. PET/CT in diagnosis of movement disorders. Ann N Y Acad Sci 2011;1228:93–108. http://dx.doi.org/10.1111/j.1749-6632.2011.06025.x.
[44] Schroeder JA, Schneider JS. GABA-opioid interactions in the globus pallidus: [D-Ala2]-Met-enkephalinamide attenuates potassium-evoked GABA release after nigrostriatal lesion. J Neurochem 2002;82:666–73. http://dx.doi.org/10.1046/j.1471-4159.2002.01010.x.
[45] Rothblat DS, Schneider JS. Spontaneous functional recovery from parkinsonism is not due to reinnervation of the dorsal striatum by residual dopaminergic neurons. Brain Res Bull 1994;34:309–12. http://dx.doi.org/10.1016/0361-9230(94)90068-x.
[46] Calon F, Rajput AH, Hornykiewicz O, Bédard PJ, Di Paolo T. Levodopa-induced motor complications are associated with alterations of glutamate receptors in Parkinson's disease. Neurobiol Dis 2003;14:404–16. http://dx.doi.org/10.1016/j.nbd.2003.07.003.
[47] Day M, Wang Z, Ding J, An X, Ingham CA, Shering AF, et al. Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. Nat Neurosci 2006;9:251–9. http://dx.doi.org/10.1038/nn1632.
[48] Brotchie J, Fitzer-Attas C. Mechanisms compensating for dopamine loss in early Parkinson disease. Neurology 2009;72:S32–8. http://dx.doi.org/10.1212/WNL.0b013e318198e0e9.
[49] Anglade P, Mouatt-Prigent A, Agid Y, Hirsch E. Synaptic plasticity in the caudate nucleus of patients with Parkinson's disease. Neurodegeneration 1996;5:121–8. http://dx.doi.org/10.1006/neur.1996.0018.
[50] Passos PP, Borba JMC, Rocha-de-Melo AP, Guedes RCA, da Silva RP, Filho WTM, et al. Dopaminergic cell populations of the rat substantia nigra are differentially affected by essential fatty acid dietary restriction over two generations. J Chem Neuroanat 2012;44:66–75. http://dx.doi.org/10.1016/j.jchemneu.2012.05.003.
[51] Joffre C, Nadjar A, Lebbadi M, Calon F, Laye S. n-3 LCPUFA improves cognition_ the young, the old and the sick. Prostaglandins Leukot Essent Fat Acids 2014;91:1–20. http://dx.doi.org/10.1016/j.plefa.2014.05.001.
[52] Darios F, Davletov B. Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. Nature 2006;440:813–7. http://dx.doi.org/10.1038/nature04598.
[53] Robson LG, Dyall S, Sidloff D, Michael-Titus AT. Omega-3 polyunsaturated fatty acids increase the neurite outgrowth of rat sensory neurones throughout development and in aged animals. Neurobiol Aging 2010;31:678–87. http://dx.doi.org/10.1016/j.neurobiolaging.2008.05.027.
[54] Ho A, Blum M. Induction of interleukin-1 associated with compensatory dopaminergic sprouting in the denervated striatum of young mice: model of aging and neurodegenerative disease. J Neurosci 1998;18:5614–29 [PMID 9671653].
[55] Sauer H, Oertel WH. Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. Neuroscience 1994;59:401–15. http://dx.doi.org/10.1016/0306-4522(94)90605-X.
[56] Politis M. Neuroimaging in Parkinson disease: from research setting to clinical practice. Nat Publ Group 2014;10:708–22. http://dx.doi.org/10.1038/nrneurol.2014.205.