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# Beneficial effects of n-3 polyunsaturated fatty acids administration in a partial lesion model of Parkinson's disease: The role of glia and NRf2 regulation

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ABSTRACT

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been widely associated to beneficial effect over different neurodegenerative diseases. In the present study, we tested the potential therapeutic effect of docohexanoic acid (DHA) and its hydroxylated derivate, DHAH, in a partial lesion model of Parkinson's disease (PD). One month before and four months after the striatal lesion with 6-OHDA was made, the animals were daily treated with DHA (50 mg/kg), DHAH (50 mg/kg), vehicle or saline, by intragastric administration. Animal groups under n-3 PUFA treatments exhibited a trend to improve in amphetamine-induced rotations and cylinder test. The beneficial effect seen in behavioral studies were confirmed with TH immunostaining. $TH^+$ fibers and $TH^+$ neurons increased in the experimental groups treated with both n-3 PUFAs, DHA and DHAH. Moreover, the n-3 PUFAs administration decreased the astrogliosis and microgliosis, in both the striatum and substantia nigra (SN), with a higher decrease of $GFAP^+$ and Iba-$1^+$ cells for the DHAH treated group. This experimental group also revealed a positive effect on Nrf2 pathway regulation, decreasing the positive Nrf2 immmunostaining in the striatum and SN, which revealed a potential antioxidant effect of this compound. Taking together, these data suggest a positive effect of n-3 PUFAs administration, and more concretely of DHAH, for PD treatment as it exhibited positive results on dopaminergic system, neuroinflammation and oxidative stress.

## 1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease, after Alzheimer disease, which nowadays affect > 5 million people worldwide. PD is characterized by the degeneration of dopaminergic neurons (Vivekanantham et al., 2015) in the *substantia nigra pars compacta* (SNpc). The resulted dopamine (DA) deficiency is the responsible of the classical parkinsonian motor symptoms: resting tremor, bradykinesia, rigidity and postural instability. However, non-motor symptoms are also present in the disease reducing health-related quality of life. These non-motor features include olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain and fatigue (Kalia and Lang, 2015; Lees et al., 2009; Chaudhuri et al., 2006; Schapira et al., 2017). Although the exact disease mechanisms underlying PD pathogenesis are not well understood, scientific evidences suggest that the nigral dopaminergic degeneration results from the convergence of different mechanism, including mitochondrial dysfunction, oxidative stress, apoptosis, excitotoxicity, altered protein handling and neuroinflammation (Gaki and Papavassiliou, 2014,Vivekanantham et al., 2015,Niranjan, 2014). Indeed, the presence of an active inflammatory response mediated by astrocytes and microglia has been long recognized but somewhat

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overlooked in PD. Reactive gliosis occurs within the area of neurodegeneration, highlighting the link between neurodegeneration and neuroinflammation (Hirsch and Hunot, 2009; Dexter and Jenner, 2013).

The most common treatments in clinical practice focus on DA replacement to control motor symptoms, however, its administration is related to disabling side effects such as; motor and non-motor fluctuations, dyskinesia and psychosis. Moreover, they are ineffective managing non-motor symptoms. The inefficacy of these treatments treating the symptomatology of the disease has aimed the research in the direction of novel therapies emphasizing neuroprotective activities (Oertel and Schulz, 2016; Hang et al., 2016). Among others, the use of antioxidants, polyunsaturated fatty acids (PUFAs) and polyphenols as nutraceuticals has gained great interest in the last years. The word "nutraceuticals" refers to compounds that derived from natural sources and the scientific data available support their beneficial role in the prevention and/or treatment of a disease. In the case of PD, different nutraceuticals have shown a positive effects managing the pathology of the disease; such as polyphenols from plant extract, food rich in vitamins (B,C,D and E) and PUFAs derived from fish oil (Zhao, 2009,Caruana and Vassallo, 2015,Chao et al., 2012, Dyall, 2015).

Omega-3 (n-3) and omega-6 (n-6) fatty acids are two major classes of PUFAS. n-3 PUFAs are essential nutrients in the development and functioning of brain and visual system. Moreover, there is growing evidence demonstrating that n-3 PUFAs remain important thorough our lifespan. The most abundant n-3 in the brain is docohexaeonic acid (DHA) that is required for normal neuronal function (Dyall, 2015). In addition, DHA is not only a structural membrane component but is also a modulator of crucial neurochemical processes, gene expression, synaptic plasticity and memory formation (Corsi et al., 2015). Recently, epidemiological studies have associated low levels of DHA consumption with a higher risk of suffering AD (Morris et al., 2003; Maclean et al., 2005). Moreover, a neuroprotective effect of DHA has been observed in different animal models of this disease (Boudrault et al., 2009). Although AD and PD have different clinical manifestations, both diseases shared mechanism of neurodegeneration: neuronal loss, deposit of insoluble protein filaments, oxidative stress, mitochondrial dysfunction and neuroinflammation (Xie et al., 2014). In fact, although much less scientific data is reported about DHA consumption and PD risk, recent epidemiological studies have suggested that high intake of unsaturated fatty acids decreases the risk of developing PD and protects from pesticides mediated toxicity (Kamel et al., 2014; de Lau et al., 2005). Moreover, in a recent clinical trial the effect of PUFAs and vitamin E coadministration was evaluated demonstrating that this cosupplementation improved the clinical evaluation of the patients, decreased C-reactive protein sensitivity and increased total antioxidant capacity (Taghizadeh et al., 2017). Analysis in a MPTP animal model of PD have also shown the beneficial effect of PUFAs protection against the neurotoxicity caused by MPTP (Bousquet et al., 2008; Bousquet et al., 2009). Despite the mechanism of these fatty acids is not properly described, numerous papers have described the effect of PUFAs enhancing neurotrophic factors release, promoting the regulation of genes associated with oxidative stress or apoptosis and decreasing the inflammatory status related with PD (Bousquet et al., 2011; Hashimoto et al., 2017).

Overall, we can conclude that there is an urgent need for the development of pharmaceuticals or nutraceuticals tools that could be implemented early to alter the natural progression of the disease. In the case of n-3 PUFAs, they are widely available and could represent an interesting nutraceutical option for PD treatment (Frèdéric Calon, 2008). In the present study, we tested DHA and its hydroxylated derivate, DHAH, which is known to impede its β-oxidation increasing its half-life in cell membrane (Torres et al., 2014). In order to prove their potential neuroprotective and neurorestorative effect, 6-OHDA partially lesioned rats received chronic administration of these two kinds of PUFAs during a total of 5 months. Behavioral and immunohistochemical studies were performed in order to prove their potential therapeutic effect in PD.

## 2. Material and methods

### 2.1. Materials

DHA and DHAH fatty acids in ethyl ester form were purchased from Medalchemy (Spain). They were aliquoted in topaz vials in $N_2$ inert atmosphere conditions ready for one unique use, in order to avoid its oxidation in the storage during the course of the experiment. Sodium phosphate dibasic ($NA_2HPO_4$), sodium phosphate monobasic ($NaH_2PO_4$), dibassic potassium phosphate ($K_2HPO_4$), potassium dihydrogen phosphate ($KH_2PO_4$), sucrose, sodium chloride (NaCl), p-formaldehyde, $H_2O_2$, xylene and Tween® 80 were obtained from Panreac (Spain). 6-hydroxydopamine hydrochloride (6-OHDA), desipriamine hydrochloride, chloral hydrate, amphetamine sulphate, carboxymethylcellulose sodium (CMC), 3,3′-diaminobenzidine (DAB), Triton X-100, sodium azide, bovine serum albumin (BSA), MetOH, mouse anti-glial fibrillary acidic protein antibody (GFAP), rabbit anti-tyrosine (TH) hydroxylase antibody, Fluoromount aqueous mounting medium and Depex (DPX) mounting medium were purchased from Sigma-Aldrich (Spain). 4′,6-diamidino-2-phenylindole (DAPI), Donkey anti-mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Donkey anti-rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 were purchased from Termofisher Scientific (Spain). Normal goat serum (NGS), biotinylated goat α-rabbit and the avidin-biotin-peroxidase complex (ABC) kit from Palex (Spain). Polyclonal rabbit Iba-1 (ionized calcium-binding adapter molecule 1) antibody from Synaptic Systems (Germany), polyclonal rabbit Nrf2 (nuclear factor erythroid 2–related factor 2) antibody from Abcam (UK) and Isofluorane Esteve from Maipe Comercial (Spain).

### 2.2. Animals

Male albino Sprague-Dawley rats (170–220 g) were housed in groups of 4 under standard laboratory conditions (22 ± 1 °C, 55 ± 5% relative humidity, and 12:12 h light/dark cycle) with food and water provided ad libitum. Every effort was made to minimize animal suffering and to use the minimum number of animals per group and experiment. Experimental protocols were reviewed and approved by the Local Ethical Committee for Animal Research of the University of the Basque Country (UPV/EHU, CEEA, ref.ES48/054000/6069). All of the experiments were performed in accordance with the European Community Council Directive on "The Protection of Animals Used for Scientific Purposes" (2010/63/EU) and with Spanish Law (RD 53/2013) for the care and use of laboratory animals.

### 2.3. 6-hydroxidopamine (6-OHDA) lesion

The 6-OHDA lesion was performed according to previous studies (Gartziandia et al., 2016). Thirty minutes before 6-OHDA injection, the rats were pre-treated with desipramine (25 mg/kg, intraperitoneal (i.p)), and then, they were anesthetized with isoflurane inhalation (1.5–2%) and mounted on a Kopf stereotaxic instrument. The lesion was generated by the injection of 3 μg/μl 6-OHDA solution into the striatum of the right hemisphere of the rats. Three injections of 2.5 μl of 6-OHDA solution (a total volume of 7.5 μl) were administered at a rate of 0.5 μl/min at three coordinates, relative to the bregma and dura, with the toohtbar set at −2.4: AP + 1.3 mm, ML +2.8 mm, DV -4.5 mm; AP -0.2 mm, ML +3.0 mm,DV -5.0 mm and AP -0.6 mm, ML +4.0 mm, DV -5.5 mm.

### 2.4. Experimental protocol

Fig. 1 shows the experimental protocol used. Before the 6-OHDA lesions were made to generate a partial lesion model of PD, the animals

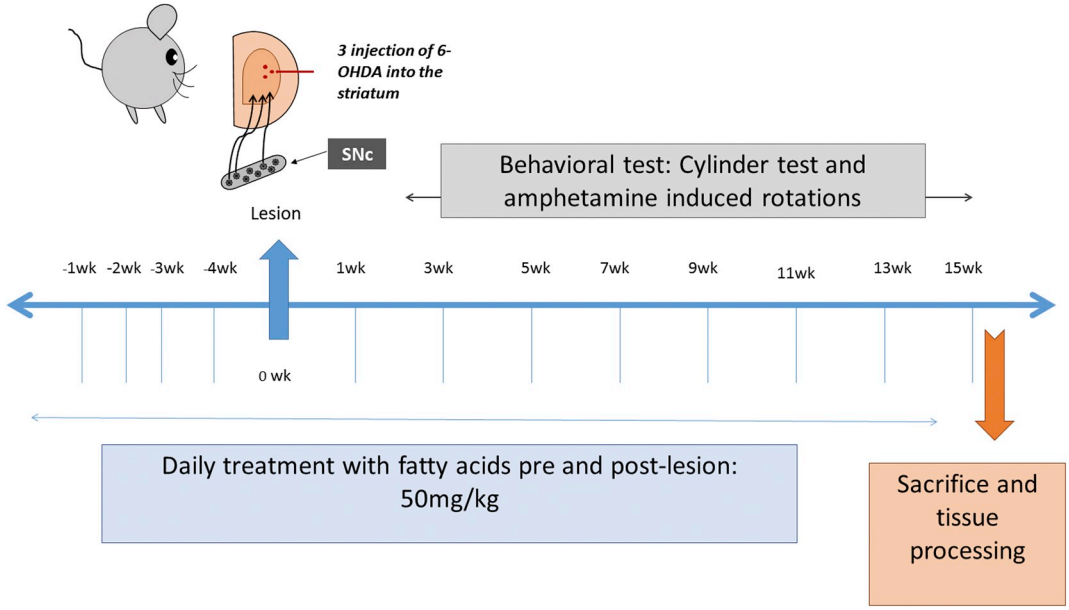


**Fig. 1.** Schematic representation of the in vivo experimental study.

were randomly divided into four groups ($n = 8$ animals, *per group*). During four weeks pre-lesion, two groups received intragastric daily treatment, with 18G gavage needle, of 50 mg/kg of DHA or DHAH diluted in an aqueous solution with 0.5% of CMC and 0.05% of Tween 80. The other two groups received; one of them vehicle (aqueous solution with 0.5% CMC and 0.05% of Tween 80) and the other one just saline. These treatments were maintained during 15 weeks after the 6-OHDA surgery and, during this period, the behavioral tests were performed every 2 weeks. 15 weeks after the lesion was made, the animals were sacrificed and their brains processed for immunohistochemical evaluation.

Animals were included in the study when showed > 3 turns per minute and < 25 turns per minute in the amphetamine-induced rotational test in the first three weeks and in the last weeks after 6-0HDA injection. In addition, this criterion was supported by TH-immunostaining, in saline and vehicle groups, indicating that those animals showed < 3 turns per minute in the beginning and in the end of the experiment did not show enough reduction of TH-immunoreactive (ir) fibers in the striatum (40% reduction in TH-ir fiber in the ipsilateral side respect to the contralateral one) to consider them as partially lesioned. On the other hand, animals showing > 25 turns per minute in the beginning of the study were also excluded because the loss of TH-ir fibers in the striatum in the end of the experimental period was also almost complete (> 90% loss of TH-ir fibers in the ipsilateral hemisphere). In short, 25% of rats were discarded according to the exclusion criteria.

### 2.5. Amphetamine induced rotational test

Three week after inducing the 6-OHDA lesion, when the lesion with toxic is stabilized, the rats were tested in the amphetamine induced rotational test. This test was repeated once every two weeks. For this purpose, D-amphetamine (5 mg/kg (i.p.) in 0.9% NaCl; Sigma-Aldrich, St. Louis, USA) was intraperitonealy administered and, after 15 min of latency, in an individual cage for each animal, the total number of full ipsilateral rotations were recorded for 90 min with an automated rotameter (Multicounter LE3806; Harvard Apparatus, Holliston, MA, USA). The results are expressed as the% of ipsilateral turns per minute.

### 2.6. Cylinder test

Forelimb use asymmetry was assessed using the cylinder test fortnightly. Rats were individually placed in a 20 cm diameter glass cylinder and allowed to explore freely. Mirrors were placed behind the cylinder to allow a 360° view of the exploratory activity. Each animal was left in place until at least 20 supporting front paw touched were done on the walls of the cylinder. The session was videotaped and later analyzed. Touches performed with the contralateral (injured side) or ipsilateral (uninjured side) front limb were counted and data are expressed as the percentage of ipsilateral placement, calculated as the following equation:

$$\%ipsilateral\ touches = \frac{ipsilateral\ paw\ placement}{ipsilateral + contralateral\ paw\ placement} * 100$$

### 2.7. Tissue preparation and postmortem analysis

The rats were transcardially perfused with 0.9% (*w/v*) NaCl and 4% (w/v) paraformaldehyde in 0.1 M PBS, pH 7.4. The brains were removed and post-fixed for 24 h in paraformaldehyde and then transferred to a 30% sucrose solution (*w/v*) in PBS 0.1 M for dehydration. After at least 3 days, brains were coronally sectioned on a freezing microtome (50 µm) and kept in PBS 0.1 M solution with sodium azide 0.1% (w/v) at 4 °C for further examination.

### 2.8. TH and Nrf2 immunohistochemistry

For TH and Nrf2 immunohistochemistry, sections were rinsed 3 times in potassium phosphate buffered saline (KPBS) (0.02 M, pH 7.1), and then the endogenous peroxidases were quenched using 3% (*v/v*) $H_2O_2$ and 10% (v/v) methanol in KPBS for 30 min (min) at room temperature (RT). After 3 rising steps in KPBS, the brain sections were preincubated in 1% (v/v) Triton X-100 with KPBS (KPBST) and NGS 5% (*v/v*) for 1 h (h) at RT to block nonspecific binding sites. Then, they were incubated overnight (ON) with rabbit polyclonal antibody anti-TH (1:1000) or rabbit polyclonal antibody anti- Nrf2 (1:400) respectively, in 5% NGS (*v/v*) KPBST at RT. After, they were rinsed twice with KPBS and once with 2.5% NGS (v/v) in KPBST. Afterwards, the sections were incubated for 2 h with a secondary biotinylated goat anti-rabbit IgG, which was diluted 1:200 in KPBST with 2.5% NGS (v/v). All sections were processed with ABC kit for 1 h, and then the reaction was visualized using DAB as the chromogen. The reaction was stopped with

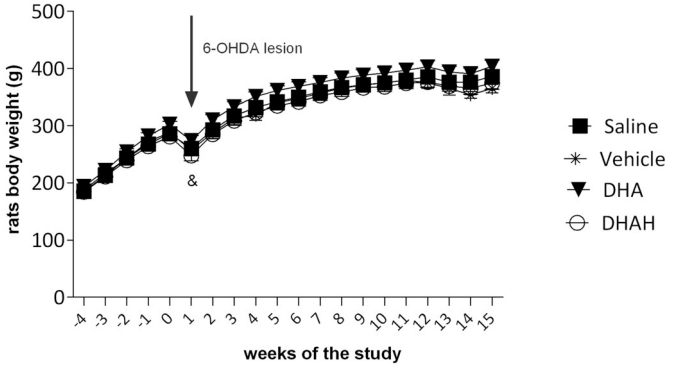


**Fig. 2.** Evolution of rats' body weight during the study. $^{\&}p < .0001$ (week 1) between all the groups; Two-way ANOVA, Tukey's multiple comparisons test.

successive rinsing steps with KPBS. Finally, the brain sections were mounted in gelatin-coated slides, dehydrated in ascending series of alcohols, cleared in xylene and coverslipped with DPX mounting medium.

### 2.9. Iba-1 and GFAP immunohistochemistry

After selecting the brain areas of the striatum and SN, Iba-1 (ionizing calcium-binding adaptor molecule 1) and glial fibrillary acidic protein (GFAP) immunohistochemistry were performed. Fixed brain sections were blocked with 2% (*w/v*) of BSA solution and 0.5% (*v/v*) Triton X-100 in PB during 1 h at RT. After rinsing, they were incubated in rabbit polyclonal antibody Iba-1 (1:1000) or mouse monoclonal anti-GFAP (1:400), respectively with 0.1% (w/v) BSA and 0.1% Triton X-100 in PB with agitation ON at 4 °C. The following day, brain slices were incubated with the secondary antibody: anti-rabbit Alexa Fluor IgG 488 (1:1000) or anti-mouse Alexa Fluor IgG 488 (1:1000), respectively, in PB with 0,1% BSA and 0,1% Triton X-100 during 2 h at RT. After three rinsing steps, the slices were incubated in DAPI (1:10.000) in PB during 10 min. Then, the slices were washed twice with PB and mounted on gelatin-coated slides and coverslipped with Fluoromount.

### 2.10. Integrated optical densitometry (IOD) of striatum

The optical density of the TH immunoreactive dopaminergic fibers in the striatum was measured using Image J win-64 Fiji and reading optical densities as grey levels. Images from section were taken with a 1200 pp. resolution digital scan (Epson). The IOD reading was corrected for background staining by subtracting the values of an area outside of the tissue from the obtained IOD of the striatum. For each animal eight slices were expressed as the percentage of IOD in the ipsilateral side respect to the contralateral non-lesioned side, which was set as 100%.

### 2.11. Number of $TH^+$ neurons in SN

$TH^+$ neuronal density in the SN was measured using a stereological tool (an optical fractionator) provided by the previously referred Mercator system. Probes of 50 × 50 μm separated by 100 μm were launched into the previously delimited area corresponding to the SN region. The counting was performed using a 40× objective. Positive cells that were present inside the probe or crossing on the right side of the X-Y axis were counted. A minimum of six histological sections per animal were used. Measurements from each slice were taken, and the mean value per animal was calculated.

### 2.12. Iba-1, GFAP and Nrf2 immunohistochemistry evaluation in striatum and SN

The analysis of Iba-1, GFAP or Nrf2 positive cells was performed by an unbiased stereology method. Images were taken using a light microscopy (Zeiss Aniolab with a Olympu 0P71 camera) at 20× magnification or a confocal microscopy (Zeiss Axiobserve Apotome 2, 20× Plan Aplochromat NA 0.8 objetive). Six slices from each animal were used to represent the areas of interest for the analysis, striatum and SN. Images were taken on the contralateral and ipsilateral side. Iba-1, GFAP and Nrf2 cells were scored as positive if their cell-body image included well defined nuclear counterstaining. The data is expressed as the percentage of the contralateral non-lesioned side, which was set as 100%.

### 2.13. Statistical analysis

All results are expressed as means ± SEM. Experimental data were analyzed using the computer program GraphPad Prism (v. 6.01, GraphPad Software, Inc.) Two-way ANOVA was used for analyzing animal body weight during experimental period and behavioral data. One-way ANOVA was used for analyze $TH^+$, GFAP and Nrf2 histological evaluation in both the striatum and SN. Student's test was applied for Iba-1 immunohistochemistry. *P values* < .05 were considered significant.

## 3. Results

### 3.1. Animal health and survival

All animals appeared healthy during the experimental period. The success of the lesion was visible as a reduction on the body weight for all experimental groups at week 1 of the study; one week after the lesion was made (week 0). As seen in Fig. 2. the variation of body weight between the four experimental groups during the study had no statistical significance, however, as previously pointed out one week after the lesion the body weight of all experimental groups decreased, being statistically significant ($^{****}p < .0001$, two-way ANOVA). The rats began the study with around 200 g, week −4, (saline: 184.9 g ± 4.0, vehicle: 187.5 g ± 4.8, DHA 193.4 g ± 4.5, DHAH: 184.2 g ± 3.5) and gained weight gradually until almost 400 g at the end point of this study, week 15 (saline: 385.9 g ± 9.2, vehicle: 364.9 g ± 7.3, DHA 403.7 g ± 10.9, DHAH: 374.7 g ± 11.2).

### 3.2. Effects of PUFAs treatment in the behavior of hemiparkinsonian rats

#### 3.2.1. Amphetamine-induced rotations

The amphetamine-induced rotations evaluation was performed in all experimental groups to study the evolution on motor performance during the treatments. The first session was made three weeks after the lesion, when the 6-OHDA lesion was stabilized. Then, consecutive sessions were made once every two weeks until the end of the study. At the very beginning of the study all experimental animals exhibited very similar values, as the study advanced an improve trend in motor performance can be seen in the groups under PUFAs treatment (Fig. 3). However, this recovery was not statically significant at any point of the study.

#### 3.2.2. Cylinder test

The cylinder test was performed in all experimental groups to study the evolution in forelimb asymmetry. As seen in Fig. 4, the groups under the PUFAs treatment exhibited a recovery tendency but this was not statically significant at any point of the study. Although a statically significance cannot be seen, the percentage of ipsilateral touches decreased with the PUFAs treatment since the beginning of the study, being lowest at week 15 (saline: 95.83% ± 3.22, vehicle;

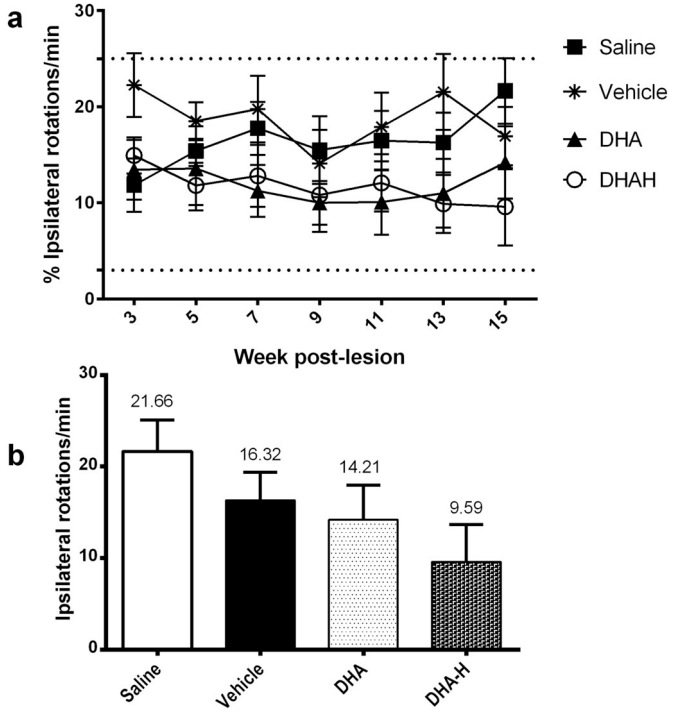


**Fig. 3.** Amphetamine induced rotations. (a) Graphical representation of the evolution on amphetamine induced rotations during the study. (b) Graphical representation of the data obtained at week 15 in the study ($p > .05$, Two-way ANOVA, Tukey's multiple comparisons test).

92.32% ± 6.19, DHA: 84.32% ± 3.83, DHAH: 86.13% ± 6.23; $p > .05$).

### 3.3. TH immunohistochemistry

Inmunohistochemical techniques were also used to analyze the efficacy of PUFAs treatments in the 6-OHDA animal model. For this purpose, at week 15 of our study the animals were sacrificed in order to examine the changes in the nigrostriatal system. Fig. 5 shows representative photomicrographs of the TH-immunostained striatum of all the different experimental groups. It is remarkable the degeneration of $TH^+$ fibers in the striatum after 6-OHDA administration in both saline and vehicle groups (saline: 23.33% ± 2 0.68, vehicle: 18.23% ± 2.91), confirming the success of the lesion. A reduction higher than 40% in TH-ir fibers in the ipsilateral side respect to the contralateral side was considered to assess the success of the lesion and confirm a partial model of PD. The administration of DHA statistically increases the density of TH fibers (DHA: 29.34% ± 3.03 vs vehicle: 18.23% ± 2.91;$^{*}p < .05$). However, as seen in the Fig. 5, this increment on $TH^+$ fibers was higher in the DHAH treated grouped (DHAH: 41.01% ± 2.93 vs saline: 23.33% ± 2.68, vehicle: 18.23% ± 2.91; $^{\&}p < .0001$).

On the other hand, the percentage of recovery of $TH^+$ positive neurons was analyzed in the SN (Fig. 6). As in the striatum, the percentage of $TH^+$ neurons decreased after 6-OHDA injection (saline: 10.70 ± 1.89, vehicle: 11.03 ± 20.3), ratifying the lesion made by the parkinsonizing agent. As in striatum, the administration of DHA increased the numbers of $TH^+$ neurons in SN (DHA: 24.48 ± 3.17 vs saline: 10.70% ± 1.89, $^{\#}p < .01$ and vehicle: 11.03% ± 2.03, $^{*}p < .05$). This augmentation of $TH^+$ neurons in SN was also observed in the DHAH- treated group (DHAH: 31.01% ± 3.32 vs saline: 10.70% ± 1.89, $^{\%}p < .001$ and vehicle: 11.03% ± 2.03,$^{\&}p < .0001$), showing a major recovery or preservation in the number of TH+ neurons than DHA-treated group. However, there was no statically significance between the administrations of the two kinds of PUFAs in the SN.

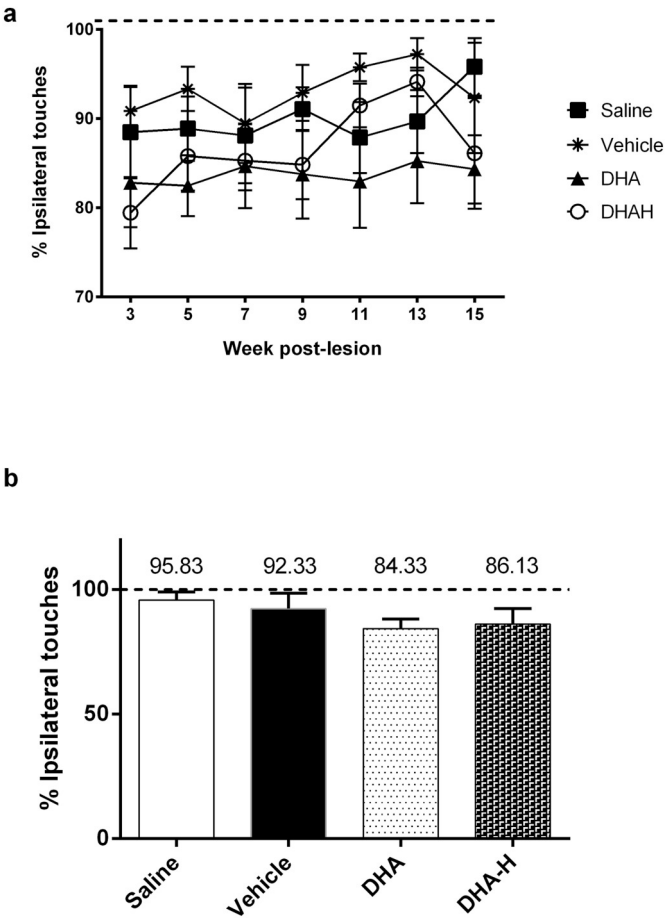


**Fig. 4.** Cylinder test (a) Graphical representation of the evolution on the percentage of ipsilateral touches for all experimental groups. (b) Graphical representation of the data obtained at week 15 in the study (p > .05, Two-way ANOVA, Tukey's multiple comparisons test)).

### 3.4. GFAP immunohistochemistry

GFAP immunohistochemistry was performed to test the ability of PUFAs treatment to modulate the neuroinflammatory component of the disease (Fig. 7.). First, the potential astroglial toxicity of 6-OHDA was confirmed in both the striatum and SN as the administration of saline and vehicle duplicated the number of $GFAP^+$ cells. The number of $GFAP^+$ cells was markedly increased in striatum; (saline: 245.4% ± 27.8 and vehicle: 259.3% ± 40.37) and in the SN (saline: 214.6% ± 21.87, vehicle: 174.0% ± 11.08). In the striatum, the administration of DHA and DHAH statically decreased the number of $GFAP^+$ cells ($^{*}p < .05$ DHA: 143.4% ± 18.44 and $^{*}p < .05$ DHAH: 129.0% ± 8.36). However, there was no difference between the administrations of these two treatments. In SN, both DHA and DHAH decreased the number of $GFAP^+$ cells, as seen in the striatum. ($^{\&}p < .0001$ DHA: 115.2% ± 3.34 vs saline: 214.6% ± 21.87) ($^{\#}p < .01$ DHA: 115.2% ± 3.34 vs vehicle: 174.0% ± 11.08) ($^{\&}p < .0001$ DHAH: 129.1% ± 8.36 vs saline: 214.6% ± 21.87); $^{*}p < .05$ DHAH: 129.1% ± 8.36 vs vehicle: 174.0% ± 11.08). The ability of these two different PUFAs to modulate the activation of astroglia in the striatum and SN was similar for both of them.

### 3.5. Iba-1 immunohistochemistry

Together with GFAP immunohistochemistry, Iba-1

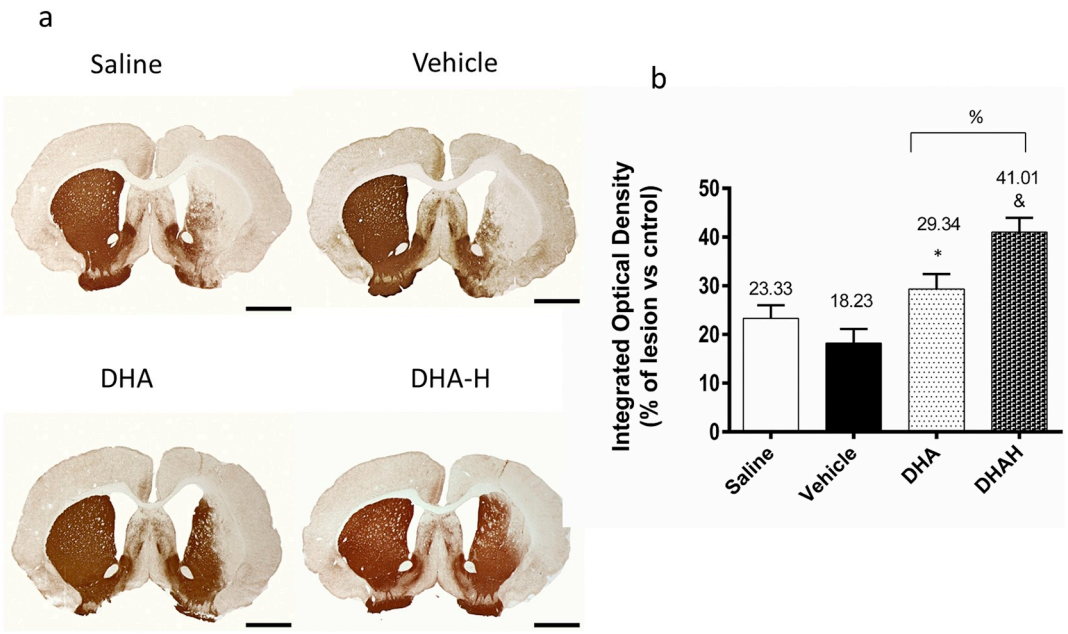


**Fig. 5.** (a) Representative photomicrographs of TH-immunostained striatum in all rat groups: saline, vehicle, DHA and DHAH (scale bar 2 mm). (b) Graph depicts the integrated optical density (IOD) of $TH^+$ fibers in the striatum of all groups. The data are shown as the mean ± SEM. ($^*p < .05$ DHA vs Vehicle, $^{\&}p < .0001$ *DHAH* vs Saline and Vehicle, $^{\%}p < .01$ DHAH vs DHA), One-way ANOVA, Tukey's multiple comparisons test).

immunohistochemistry was realized to test how the PUFAs oral administration modulate other component of neuroinflammation in PD, the activated microglia cells (Fig. 8.). As seen in GFAP immunohistochemistry, the injection of 6-OHDA led to an increase of Iba-$1^+$ cells in both the striatum and SN in non-treated groups, saline and vehicle, confirming the ability of this neurotoxin to increase the percentage of activated microglial cells (saline: 214.9% ± 19.62, vehicle: 221.2% ± 23.89 in the striatum; and saline: 191.5% ± 11.27, vehicle: 177.3% ± 10.25 in SN). In the striatum, the DHA administration led to a statistically decrease (142.7% ± 11.83 vs saline and vehicle, $^{\#}p < .01$). This reduction was even higher in the DHAH treated group (110.4% ± 9.20 vs saline and vehicle,$^{\&}p < .0001$), with a significance decrease when compared with DHA treated group ($^*p < .05$). In the case of SN, they are similar to that seen in the striatum. The saline and vehicle groups exhibited the highest percentages of activated microglial cells (saline: 191.5% ± 11.27, vehicle: 177.3% ± 10.25) and the groups treated with PUFAs nutraceuticals statically decrease the level of activated microglial cells in this brain area (DHA: 122.8% ± 7.59 vs saline and vehicle, $^{\&}p < .0001$ and DHAH: 113.6% ± 3.76 vs saline and vehicle, $^{\&}p < .0001$). However, unlike in the striatum, there was no difference between these two groups.

### 3.6. Nrf2 immunohistochemistry

Nrf2 protein activation was evaluated in order to determine the ability of DHA and DHAH administration to modulate another well-

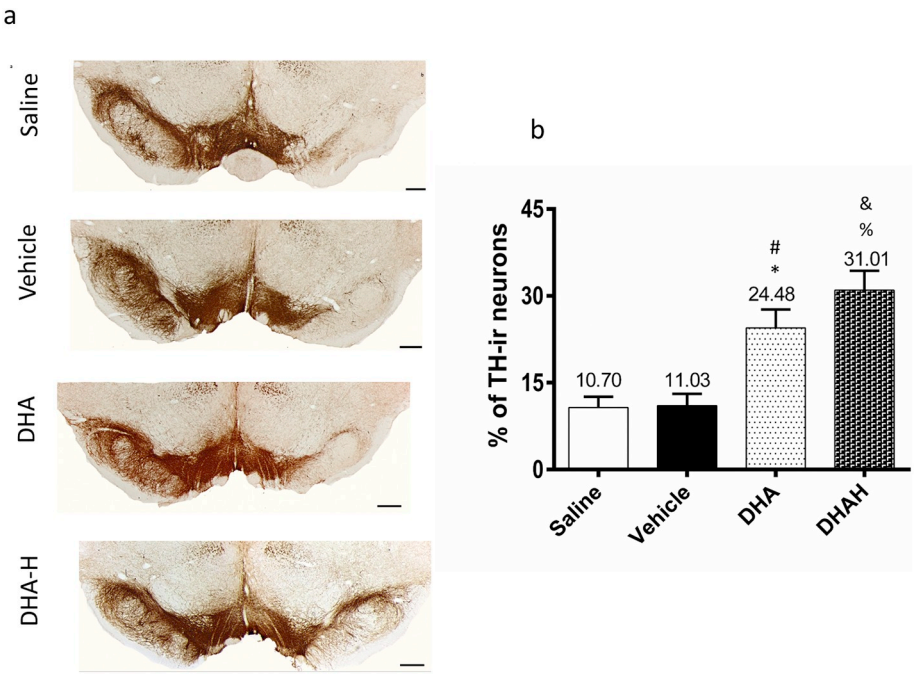


**Fig. 6.** (a) Representative photomicrographs of TH immunostain in SN in all: saline, vehicle, DHA and DHAH (scale bar 500 µm). (b) $TH^+$ neurons in SN (%). The data are shown as the mean ± SEM. ($^{\&}p < .05$ DHA vs vehicle, $^*p < .01$ DHA vs saline, $^{\&}p < .0001$ DHAH vs saline $^{\%}p < .001$ DHAH vs vehicle), One-way ANOVA, Tukey's multiple comparisons test).

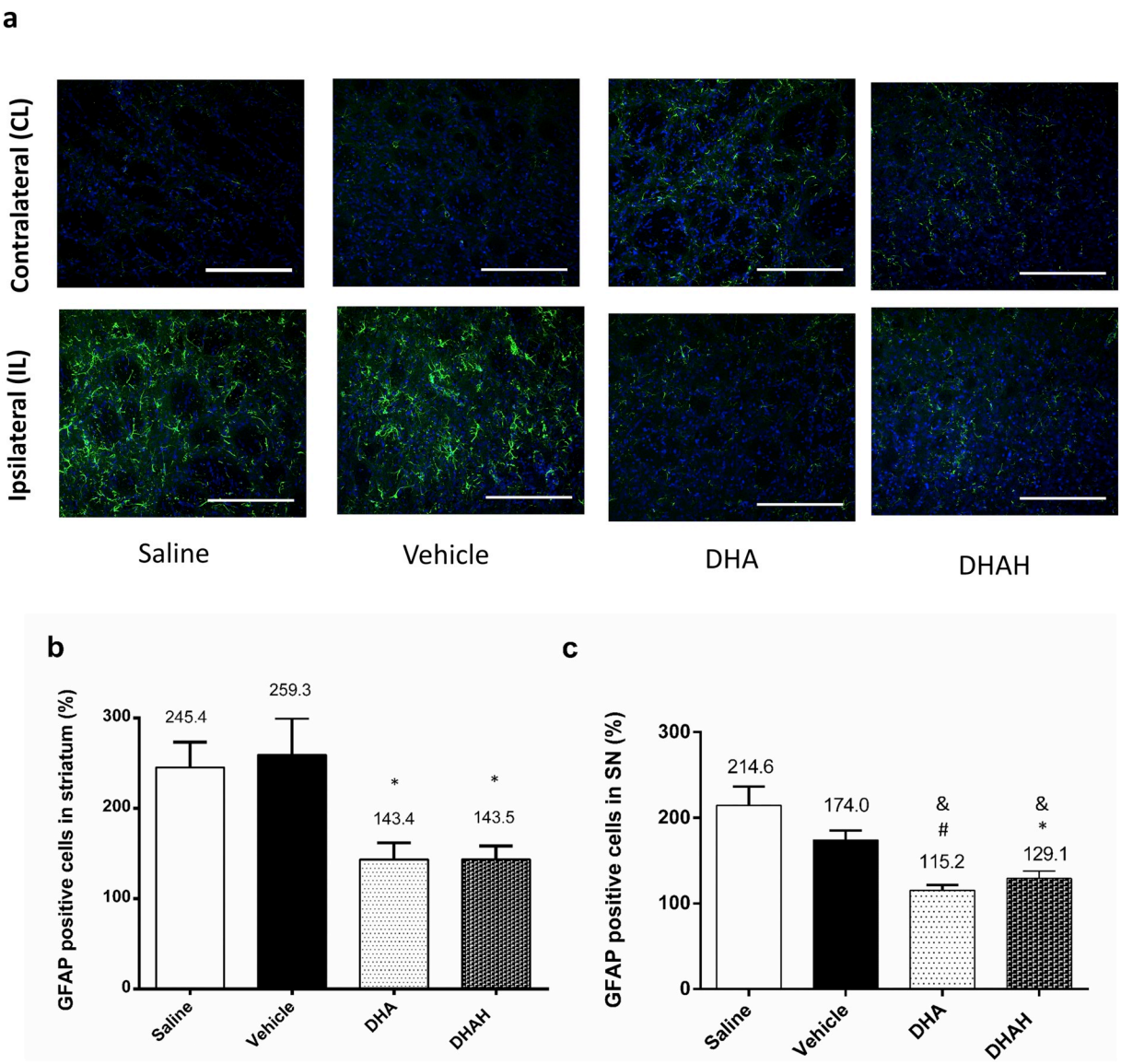


**Fig. 7.** Histological evaluation of GFAP$^+$ cells in both the striatum and SN (a) Representative GFAP immunophotographs of all rat groups in the striatum (saline, vehicle, DHA and DHAH) (scale bar 10 μm). (b) GFAP$^+$ cells in the striatum of all rat groups (%).The data are shown as the mean ± SEM. ($^*p < .05$ DHA vs saline and vehicle) ($^*p < .05$ vs saline and vehicle). (c) GFAP$^+$ cells in SN of all rat groups (%).The data are shown as the mean ± SEM. ($^{\&}p < .0001$ DHA vs saline, $^{\#}p < .01$ DHA vs vehicle) ($^{\&}p < .0001$ DHAH vs saline, $^*p < .05$ DHAH vs vehicle), One-way ANOVA Tukey's multiple comparisons test). Blue stained for nucleus and green stained for GFAP$^+$ cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

known feature of the disease, the oxidative stress (Fig. 9.). The injection of 6-OHDA to generate the PD model almost duplicate the expression of this marker in the control groups (saline and vehicle-treated groups) in both the striatum (saline: 176.6 ± 15.44, vehicle: 162.0 ± 12.68) and SN (saline: 180.4% ± 8.56, vehicle: 163.9% ± 7.34). In this case, the intragastric administration of DHA did not led to a statistically decrease of the expression in this protein neither in the striatum (133.9% ± 8.75) nor in SN (137.4% ± 4.72). Nevertheless, the group treated with DHAH exhibited better results with a statically decrease of Nrf2$^+$ cells in both the striatum (115.3% ± 6.62 vs saline $^{\#}p < .0001$ and vehicle $^*p < .001$) and in SN (120.6% ± 4.54 vs saline $^{\&}p < .0001$ and vehicle $^{\%}p < .001$).

## 4. Discussion

PD is a complex neurodegenerative disorder with multiple clinical manifestations and complex cellular and molecular pathology. Current available treatments based on traditional pharmacotherapeutic concepts are far from optimal. That is why in the last years scientists and pharmaceutical industry slowly, but progressively moved away from classical targets of pharmacotherapy to new compounds with neuroprotective properties to slow down the progression of the disease (Oertel and Schulz, 2016; Kalia and Lang, 2015). Accordingly, nutraceuticals such as flavonoids, vitamins, or n-3 PUFAs have gained the attention of the scientific community due to their potential effects modulating the pathological hallmarks of the disease: oxidative stress, neuroinflammation, mitochondrial dysfunction and excitotoxicity (Sutachan et al., 2012, Magalingam et al., 2015,Dutta and Mohanakumar, 2015,Frèdéric Calon, 2008). Moreover, recently the hydroxylation of polyunsaturated fatty acids have been proven to increase the half-life of its naturally analog since they are not degraded by β- oxidation but by α- oxidation pathway. Therefore, the therapeutic effects of these hydroxylated molecules maybe increased (Ibarguren et al., 2014; Teres et al., 2012).

In this research article, we studied the beneficial effects of DHA and DHAH, a hydroxylated derivate of DHA. Up today, the beneficial effects

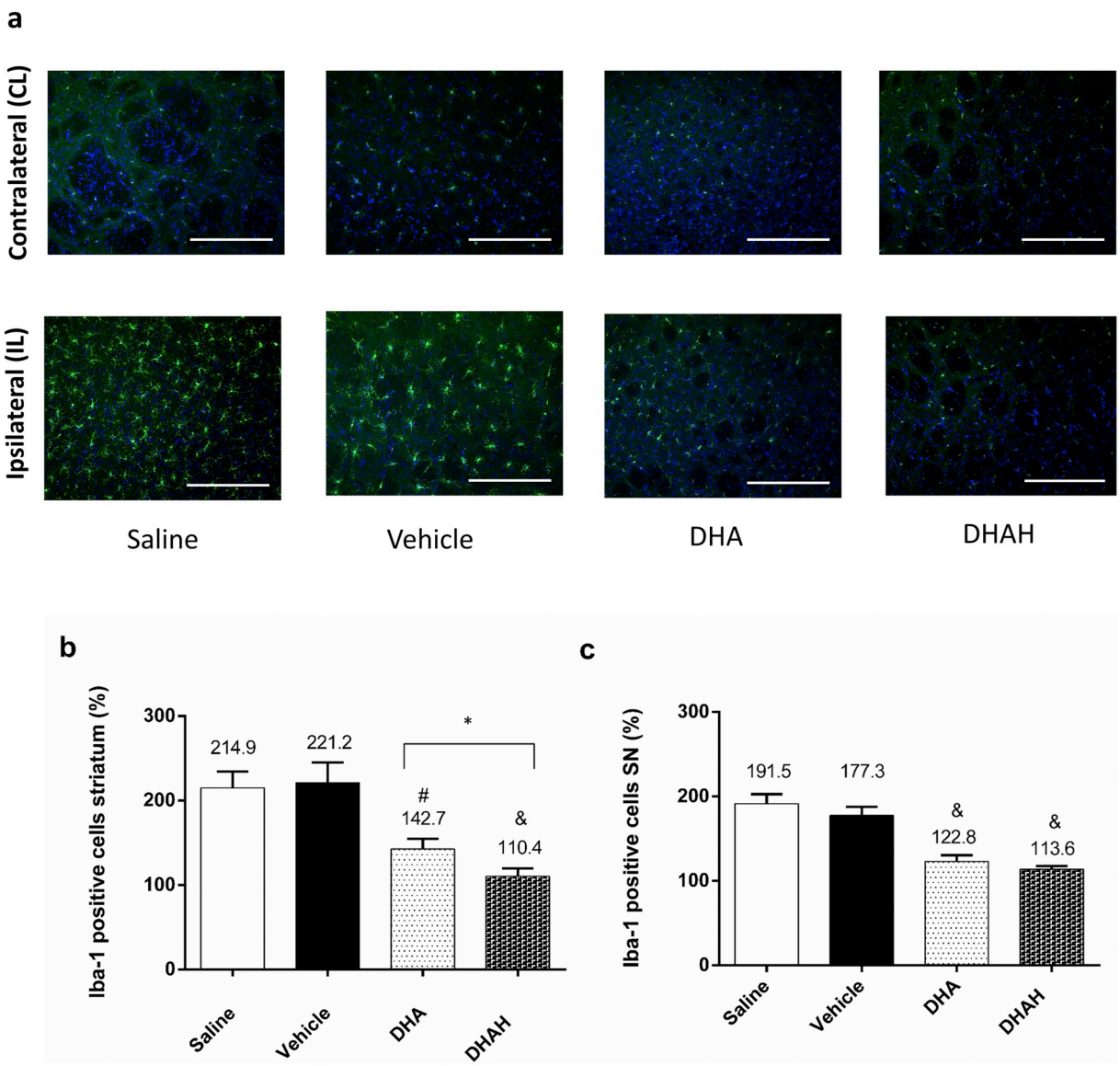


**Fig. 8.** Histological evaluation of Iba-1$^{+}$ cells in both the striatum and SN. (a) Representative Iba-1 immunophotographs of all rat groups in the striatum (saline, vehicle, DHA and DHAH) (scale bar 10 μm)). (b) Iba-1 $^{+}$ cells in the striatum of all rat groups (%). The data are shown as the mean ± SEM. ($^{\#}p < .01$ DHA vs saline and vehicle) ($^{\&}p < .0001$ DHAH vs saline and vehicle) ($^{*}p < .05$ DHAH vs DHA). (c) Iba-1 $^{+}$ cells in SN of all rat groups (%). The data are shown as the mean ± SEM. ($^{\&}p < .0001$ DHA vs saline, and vehicle) $^{\&}p < .0001$ DHAH vs saline and vehicle), unpaired Student's *t*-test. Blue stained for nucleus and green stained for Iba1$^{+}$ cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of DHA in the treatment of NDs have been described in the scientific literature (Dyall, 2015; Bousquet et al., 2011). Although the mode of action of DHA is not yet understood, numerous research papers have focused on the effects of DHA on cell membrane, concretely on lipid raft domains, which recently have been reported to be altered in NDs (Sonnino et al., 2014; Stillwell et al., 2005; Stillwell and Wassall, 2003). In the present study we studied these two kinds of PUFAS to elucidate if this molecule would equalize or increase, the beneficial effects already know for DHA in PD; testing the premise of an increase half-life and therefore, a therapeutic effect of this hydroxylated molecule. For achieving this purpose, these two kinds of n-3 PUFAs were evaluated in a 6-OHDA animal model of the disease.

6-OHDA-induced toxicity is extensively used to induce PD in murine animals. This toxin when injected in the striatum produces retrograde degeneration of nigrostriatal neurons. The lesion obtained with 6-OHDA is highly reproducible, which represents a considerable added value when new therapeutic strategies are to be investigated and clear neuroprotective effects must be demonstrated (Blandini and Armentero, 2012; Dauer and Przedborski, 2003). In the present study, amphetamine induced rotation was performed to evaluate the neuroprotective effect of n-3 PUFAs. First, the suitability of this animal model was assessed as in saline and vehicle treated groups the ipsilateral rotations per minute was higher than 10–15 turns per minute (Dunnett and Lelos, 2010). In the present study, the administration of nutraceuticals did not decrease ipsilateral induced rotations treatment and neither did the ipsilateral front paw touches in the cylinder test. Although a recovery trend can be seen in behavioral studies, these differences were not statically significant at any point of the study. The lack of statistical significance may be due to plasticity or compensatory mechanism, which may interfere with obtained rotational data. (Deumens et al., 2002) Indeed, the present work was a long study (15 weeks) and the behavioral tests were repeated several times, favoring the emergence of these processes (Schallert et al., 2000). In previous studies with chronic administration of n-3 PUFAs, similar results were observed; without a statistical difference in behavioral tests (Barros et al., 2017,Delattre et al., 2010,Coulombe et al., 2016). In any case, the recovery trend saw

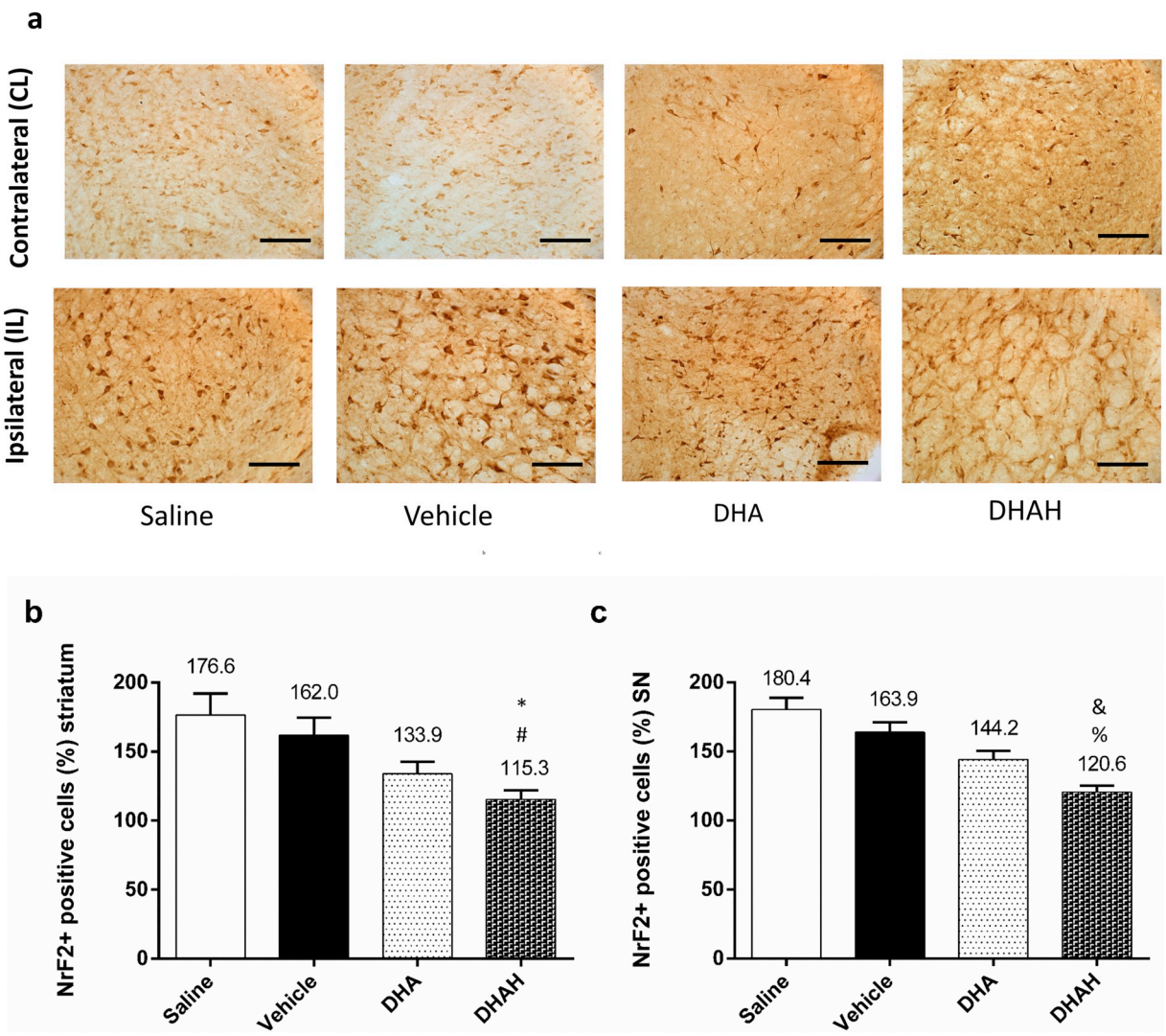


**Fig. 9.** Histological evaluation of Nrf2$^+$ cells in both the striatum and SN. (a) Representative Nrf2 immunophotographs of all rat groups in the SN (saline, vehicle, DHA and DHAH) (sale bar 2 μm) (b) Nrf2 $^+$ cells in the striatum of all rat groups (%).The data are shown as the mean ± SEM. ($^{\#}p < .01$ DHAH vs saline; $^{*}p < .05$ DHAH vs vehicle). (c) Nrf2 $^+$ cells in SN of all rat groups (%). The data are shown as the mean ± SEM. ($^{\&}p < .0001$ DHAH vs saline) ($^{\%}p < .001$ DHAH vs vehicle), One-way ANOVA, Tukey's multiple comparisons test.

in behavioral tests was confirmed with TH immunohistochemistry studies, supporting the beneficial effect of n-3PUFAs on PD. The DHAH supplementation increased up to 30% the density of TH$^+$ fibers. Accordingly, the TH$^+$ nigral neurons were higher in this experimental group. Similar results were obtained in previous studies with DHA supplementation supporting our finding (Bousquet et al., 2008; Ozsoy et al., 2011; Tanriover et al., 2010). Moreover, in this work, we confirm not only the neuroprotective and neurorestorative effect of DHA but also, of its hydroxylated derivate, DHAH, which had shown before positive effects in AD treatment; improving behavioral tests and decreasing Aβ oligomers and tau protein phosphorylation in cellular and animal models (Torres et al., 2014; Fiol-deRoque et al., 2013). The potential mechanism previously explained about the hydroxylation of these fatty acids may explain the differences observed in TH immunostaining for the two different groups of PUFAs.

On the other hand, the results obtained until today for PUFAs and PD treatment, have focused, in general, on behavioral studies, TH$^+$ immunostaining and lipid peroxidation. Indeed, much less scientific data is published relating PUFAs treatment and its effect in neuroinflammation. n-3 PUFAs are known to have an anti-inflammatory effect, (Calder, 2015) and its administration may decrease the reactive gliosis shown in PD. Actually, the role of glia and neuroinflammation in PD has been disclosed as a potential target for neuroprotection (Hirsch and Hunot, 2009). Up to now, post-mortem studies provided evidence of neuroinflammation in PD with an increase in activated microglia and astroglial cells in the SN of PD patients (McGeer et al., 1988; Damier et al., 1993). The injection of 6-OHDA led to an increase of Iba-1$^+$ and GFAP $^+$ cells in both the striatum and SN. These data is in line with previous studies where a clear astrogliosis and microgliosis have been seen in this animal model (Long-Smith et al., 2009, Kitamura et al., 2010,Henning et al., 2008). The DHA and DHAH administration decreased the activated astroglial cells, reaching almost the values seen in the contralateral side. Moreover, the results of DHA and DHAH modulating astrogliosis were similar to those seen in Iba-1 immunohistochemistry with a clear decrease of Iba-1$^+$ cells after n-3 PUFAs administration. The exact mechanism by which n-3 PUFAs mediated their anti-inflammatory properties are not still elucidated. Moreover, it is not clear if n-PUFAs inhibit glia activation directly or indirectly after enzymatic conversion to a variety of bioactive mediators. As previously pointed out, DHA is a major component of cell membrane having the ability to activate several interlinked pathways that may account their effects on cells. Among others, it is known the influence of n-3 PUFAs inhibiting the effects mediated by proresolving factors such as IL-1β, downregulating the expression of several enzymes

of arachidonic acid cascade in the brain or modifying membrane fluidity and therefore, inhibiting cytokine production or membrane ion channels (Sun et al., 2017, Hjorth and Freund-Levi, 2012,Bazinet and Laye, 2014). Indeed, the exogenous administration of DHA decreasing astrogliosis and microgliosis has been previously described in both in vitro and in vivo studies with different models of neuroinflammation (Paterniti et al., 2014; Harvey et al., 2015; Heras-Sandoval et al., 2016; Chang et al., 2015). Overall, the results obtained in this study confirmed this data and revealed a positive effect of DHAH supplementation decreasing astroglial and microglia positive cells and therefore, normalizing the activity of glia in PD injured brain.

Furthermore, in the las years, there has been a growing interest in the role of Nrf2 pathway in neurodegenerative diseases. The Nrf2 system is compromised by increased age and aging is the main risk factor for all neurodegenerative diseases, including PD. Moreover, as oxidative stress is a hallmark of PD, antioxidant therapy has been proposed as a reasonable therapeutic approach (Koppula et al., 2012). Under healthy conditions, the Nrf2-antioxidant response element (ARE) pathway is critical in order to maintain homeostasis. However, when there is an exposure to reactive oxygen species (ROS), Nrf2 dissociates from cytosolic and translocates to the nucleus where it binds to ARE which transcriptionally drive the expression of several detoxifying and antioxidant genes (Lim et al., 2014; Sandberg et al., 2014). In the case of PD, immunostainning for Nrf2 was observed in dopaminergic neurons in the SN of PD patients (Ramsey et al., 2007). The activation of Nrf2 pathway after 6-OHDA exposure has seen both in vivo and in vitro studies (Siebert et al., 2009; Zhang et al., 2014; Jakel et al., 2005). The present study confirmed this data since saline and vehicle experimental groups exhibited positive Nrf2 immunostaining. Although scientific data have reported a decrease on $Nrf2^+$ cells after DHA administration, we were not able to see that results neither in the striatum nor in SN. However, the studies were performed in cell culture or in other animal models of neuroinflammation such as ischemia or traumatic brain injury (Ozkan et al., 2016). In this case, only the DHAH administration had a statically decrease after the chronic administration of this n-3 PUFA, maybe highlighting a higher potential antioxidant capacity for this compound.

Altogether, these results support the use of n-PUFAs, and more concretely the DHAH as a nutraceutical option for PD treatment. We demonstrated a positive effect of n-3 PUFAs supplementation on DAergic system, neuroinflammation and oxidative stress. As neurodegenerative diseases prevalence will continue to rise in the next decades, neuroprotective strategies have been widely studied and n-3 PUFAs have demonstrated to be a promising therapeutic approach. Moreover, those evidences have increased the development of new formulations and synthetic modifications from natural sources of n-3 PUFAs, such as DHAH. The synthetic modification of DHA to convert in DHAH have showed to increase the beneficial effects already known about DHA. Continue in this line of research should hopefully bring new nutraceuticals or pharmaceuticals options for an effective treatment of PD. However, larger clinical trials should be made in order to establish a clearer link between n-3 and PD. On the other hand, more studies are necessary to determine the action mechanism of n-3 PUFAs in PD, to stablish the therapeutic effective dosage and the state of the disease in which this treatment should have a beneficial effect.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2018.10.001.

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