Research report

Ethyl-eicosapentaenoate (E-EPA) attenuates motor impairments and inflammation in the MPTP-probenecid mouse model of Parkinson’s disease

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A B S T R A C T

Parkinson’s disease (PD) is a neurodegenerative disorder, characterized by hypokinesia, but also mood and cognitive disorders. Neuropathologically, PD involves loss of nigrostriatal dopamine (DA) and secondary non-dopaminergic abnormalities. Inflammation may contribute to PD pathogenesis, evident by increased production of pro-inflammatory cytokines. PD onset has been positively associated with dietary intake of omega-(n)-6 polyunsaturated fatty acids (PUFA). On the other hand, omega-(n)-3 PUFA may benefit PD. One of these n-3 PUFA, eicosapentaenoic acid (EPA), is a neuroprotective lipid with anti-inflammatory properties, but its neuroprotective effects in PD are unknown. Thus, we presently tested the hypothesis that EPA can protect against behavioral impairments, neurodegeneration and inflammation in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-probenecid (MPTP-P) mouse model of PD. MPTP-P injections caused hypokinesia in the rotorod and pole test, hyperactivity in the open field, and impaired mice on the cued version (procedural memory) of the Morris water maze. MPTP-P caused a loss of nigrostriatal DA and altered neurochemistry in the frontal cortex and hippocampus. Furthermore, striatal levels of pro-inflammatory cytokines were increased, while the brain n-3/n-6 lipid profile remained unaltered. Feeding mice a 0.8% ethyl-eicosapentaenoate (E-EPA) diet prior to MPTP-P injections increased brain EPA and docosapentaenoic acid (DPA) but not docosahexaenoic acid (DHA) or n-6 PUFA. The diet attenuated the hypokinesia induced by MPTP-P and ameliorated the procedural memory deficit. E-EPA also suppressed the production of pro-inflammatory cytokines. However, E-EPA did not prevent nigrostriatal DA loss. Based on this partial protective effect of E-EPA, further testing may be warranted.

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1. Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder, characterized by motor impairments and neuropsychiatric symptoms, including anxiety and depression. Progressive degeneration of nigrostriatal dopaminergic neurons leads to the loss of dopaminergic terminals and dopamine (DA) in the striatum. Neurodegeneration may also extend to non-dopaminergic and extra-nigrostriatal systems, which may underlie the complex symptomatology of PD. Indeed, cognitive impairment and dementia may occur in PD as well [1]. Although Parkinsonian cognitive impairment is typically characterized by fronto-striatal executive dysfunction, atrophy in the hippocampus, an important brain region in memory processing, has been demonstrated [2].

Among various pathogenesis, neuroinflammation may be important in PD [3]. Activated microglia have been reported in PD and the nigrostriatal system has the brain’s highest density of microglia [3,4]. Activated microglia can produce pro-inflammatory cytokines, which may be directly neurotoxic [3]. Furthermore, enzymes involved in the arachidonic acid (AA) cascade are activated in PD [5,6] and these enzymes can be triggered by cytokines [7]. Cytosolic phospholipase A2 (cPLA2) catalyzes the release of omega-(n)-6 polyunsaturated fatty acid (PUFA) AA from neuronal membranes, and further modification of AA by cyclooxygenase 2 (COX-2) leads to the production of oxidants and
potentially anti-inflammatory eicosanoids [8]. Increased intake of n-6 in proportion to omega-(n-3) PUFA, as in Western diets, increases the availability of n-6 substrate for COX-2, which may be linked to the increased prevalence of neurodegenerative disorders such as PD [9]. Furthermore, administration of Parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to mouse brain slices increased AA content and cPLA2 expression [10], further suggesting involvement of the AA metabolic pathway and inflammation in PD pathogenesis.

On the other hand, therapeutic benefit of omega-(n-3) PUFA in neurodegenerative disorders has been reported [11]. By influencing biophysical properties of membranes and metabolism to eicosanoids, n-3 PUFA modulate neurochemistry, signal transduction and gene expression [11,12]. Well documented are their anti-inflammatory effects. Eicosapentaenoic acid (EPA), can suppress the formation of inflammatory eicosanoids, by competing with AA for COX-2 [12]. Furthermore, by activating peroxisome proliferator-activated receptors (PPARs), n-3 PUFA can suppress the transcription of inflammatory genes, including COX-2 [13,14]. These pleiotropic effects of n-3 PUFA may explain the behavioral (motor, mood and cognition) changes observed with modulation of brain n-3 content by dietary restriction or supplementation [15].

As current therapies are limited to symptom reduction [16], treatment for PD is urgent. Recent studies [17–19] demonstrating neuroprotective effects of n-3 PUFA in experimental PD used a fish-oil compound largely containing docosahexaenoic acid (DHA). While DHA is the major n-3 PUFA of neuronal membranes and EPA is only a trace component, the latter is known to be a neuroactive and protective lipid [20,21]. EPA is extensively utilized to modulate cellular functions, which may explain its lower quantity in neuronal membranes [22]. EPA also prevented interleukin-1β (IL-1β)-induced inflammation, anxiety, depression and memory-impairment in rodents [23–26]. Furthermore, we previously demonstrated that EPA has some modest neuroprotective action against experimental PD induced by MPP+ in mouse brain slices [10]. To obtain more elaborate in vivo evidence, we presently tested the hypothesis that a diet enriched in EPA can attenuate behavioral impairments, neurodegeneration and inflammation induced by MPTP in mice. We used the MPTP-probenecid (MPTP-P) model, since it causes the most profound striatal and extra-striatal neuropathology as well as inflammation [27,28].

2. Material and methods

2.1. Animals

All experimental procedures were carried out in agreement with the Canadian Council on Animal Care (CCAC), and approved by the Animal Care Committee of the University of Prince Edward Island. Male C57BL/6 mice (Charles River, Canada), 6 weeks of age, were habituated for 1 week to the animal colony and had free access to food (regular chow, PMI Richardson, Indiana, US) and water at all times. Animals were housed individually in a 12–12 light–dark cycle with room temperature at 22 ± 1°C.

2.2. Experimental procedures and methods

For an overview of the experimental design, see Fig. 1.

2.2.1. Behavioral pre-training

One week following normal habitation, the mice were pre-trained on the rotorod and pole-test until ceiling performance was reached. As evident in Fig. 1, the time between pre-training and experimental sessions was substantial, at 79 days. While some may argue that mice will forget the acquired task, we found this not to be the case, as saline treated (control) mice still performed near maximum level (rotorod distance moved and pole test total time) on the first day of post-MPTP experimentation. Since we were interested in the effect of experimental treatment on motor performance “per se” and not motor learning, we considered it important to pre-train mice prior to any experimental manipulation.

2.2.2. Experimental diets

Immediately following pre-training, the regular rat chow was replaced with a diet consisting of regular chow supplemented with 0.8% ethyl-eicosapentaenoate (E-EPA) (Amarin Neuroscience Ltd, UK) (N = 20) or palm oil (Sigma, Canada) (N = 20). The latter served as control diet, as it ensured comparable caloric values, contains very low amounts of linoleic acid (C18:2, n-6) and no n-3 fatty acids [24]. The nutritional composition and procedures of the diets are outlined in Table 1. The feeding period prior to MPTP-P injections was 6 weeks, but the diet continued until the animals were sacrificed. It was previously determined in C57BL/6 mice that 6 weeks is the sufficient feeding time to raise brain EPA levels significantly [10].

2.2.3. MPTP-P administration

100 mg MPTP hydrochloride (Sigma, Canada) was dissolved in 20 mL sterile saline, rendering a 5 mg/mL solution that was injected. Probenecid (Sigma, Canada), is an adjuvant drug that reduces the renal excretion of MPTP and thus its excretion from the body, thereby prolonging the neurotoxic effect of MPTP during the 3.5 days inter-injection interval. It was dissolved in 16 mL 0.1N NaOH, which was stabilized with 1 M Tris–HCl (pH 6.8) to a pH of 7.4 and final volume adjusted to 32 mL with ddH2O [27]. Probenecid solutions were prepared freshly for each injection. The chronic MPTP-P dosing regimen was adapted from previous protocols [28] and conducted as described in Ref. [27]. In brief, mice were administered 10 injections of MPTP 25 mg/kg s.c. combined with probenecid 250 mg/kg i.p. (n = 20), or saline s.c. combined with probenecid 250 mg/kg i.p. (n = 20), 3.5 days apart. Thus, there were four experimental groups: (1) Palm and MPTP-P (MP); (2) E-EPA and MPTP-P; (3) Palm and saline (P); (4) E-EPA and saline (E). Probenecid by itself does not affect neurochemistry or behavior [28], an extra experimental group to investigate the effect of probenecid was not carried out in the present study.

2.2.4. Behavior testing

A test battery similar to Ref. [27] was used to assess the hypokinetic, mood and cognitive aspects of Parkinsonism. All tests were done between 9 am and 2 pm under normal animal room lighting. The rotorod (hypokinesia) and open field (hypokinesia and anxiety) were tested on the second and third day after the last injection respectively. The pole test (hypokinesia) was performed on the fourth day following injection. For spatial learning and memory, the Morris water maze was carried out one week after injections.

2.2.4.1. Rotorod

The protocol was modified from previous procedures [29]. The animals were positioned on the rotating (ITT Life Science, CA, USA), which was programmed to rotate with linearly increasing speed from 1 rpm to 30 rpm in 300 s. Automatic sensors captured when animals fell off the rod and the total distance moved (m) automatically calculated. During pre-training, mice performed 4 trials a day and rested 1 min between trials. On the post-MPTP-P testing day, the average of total distance moved on 5 successive trials was measured. Between each trial, animals rested for 1 min.

2.2.4.2. Open field

A white plastic rectangular box (70 cm × 30 cm × 20 cm) with 5 cm² squares drawn on the bottom was used for assessment of mouse locomotor and anxiety-related behavior in a bright, open environment, as previously described [27]. The mouse was placed in the center of the open field. Grid crosses (total distance moved), rearing and grooming were manually counted for 5 min, in normal room lighting (no extra bright light source was placed immediately above the apparatus). Video recordings of each session were used to measure the percentage of time spent in the center of the arena, which is a measure of anxiety [30]. The environment was cleaned with 70% alcohol and water between trials. Each animal performed only 1 trial.

2.2.4.3. Pole test

The method was adapted from Ogawa et al. [31]. The pole test consisted of a 50-cm high wooden pole, 0.5 cm in diameter, wrapped in gauze to prevent slipping and the base position in the home cage. A rubber ball was placed on top of the pole to prevent animals from sitting on the top and to help position the animals on the pole (by sliding the forepaws over the ball and holding the animal by the tail). The time that animals required to climb down the pole was measured. If the mouse did not descend in 60 s, it was guided. During pre-training as well as post-MPTP-P sessions, each animal performed 3 successive trials, with a 5 min inter-trial interval. The average of the three trials was taken for statistical analyses.

2.2.4.4. Morris water maze

The method used for the Morris water maze was adapted from a more recent version [32]. Briefly, the water tank (1 m in diameter, with 40 cm high walls) was divided into 4 equally sized quadrants and designated as north (N), west (W), south (S), and east (E). Water was made opaque by the addition of water-soluble and non-toxic tempera paint (Funstuff) and temperature maintained at 23 ± 1°C. A security camera was mounted above the center of the tank, and swimming trials were recorded. One day prior to testing, animals were habituated to the water by swimming for 1 min without the escape platform. During the testing days, mice started each trial facing the rim of the pool and were given 60s

to complete a trial. On the first testing day, mice were trained to locate and escape on a Perspex escape platform (diameter 9 cm) that was made visible (cued version of the test) by elevating it slightly above the water and placing a brightly colored object on top of it (flag). Mice were given 4 trials and with each trial, the location of the platform changed to a different quadrant in a pseudorandom order. The purpose of this cued test was to assess procedural memory (habit learning) as well as the motivation of the mice to escape [33,34]. It also offered mice practice to escape on the platform. During the subsequent three testing days (spatial version of the test), the platform was submerged 0.5 cm below the water surface and the animals trained to locate the hidden platform. Again, mice were given 4 trials, with inter-trial times of approximately 5 min. The submerged platform remained in the same position (in the middle of one of the four quadrants), but the starting quadrant of the mice was pseudorandomly changed with each of the four trials. Those mice that failed to reach the platform within 1 min were guided towards it and then permitted to stay on for 15 s. The main dependent measure during cued and spatial training was escape latency (s). On the fourth day, the platform was removed after the fourth trial. During this probe trial, the swimming behavior was recorded over 60 s, while the mouse searched for the removed platform. The percentage of search time in the correct quadrant (the one that contained the platform) and the total number of crossings of the exact position where the platform was located (annulus) were measured. Furthermore, swimming speed and the total number of quadrant entries (each time an animal passed the boundaries of a quadrant, an entry was counted) were measured. In post hoc analyses from videorecorded material, swimming speed was measured as pixels/s with Mousetracker version 1.0 by Adriano Tort.

2.2.5. Sacrifice and biochemical analyses

Following the Morris water maze, the animals rested for 2 days. On the next day, animals were sacrificed by cervical dislocation. Brains were rapidly removed and the striatum, frontal cortex, hippocampus and midbrain dissected on ice. For analyses of neurochemistry and brain cytokines, brain samples of one hemisphere were sonicated (Misonix Inc., XL2000, NY, USA) in ice cold lysis buffer (1 L Contained 8.8 l mg-1 ascorbic acid, 8.8 mg; 9.3 l 70% HClO4, and 100 mg EDTA), centrifuged at 10,000 x g for 25 min at 4 °C and stored in −20 °C. These samples were also used for BCA protein assay (Sigma, Canada, #QPBCA) to measure total protein content in the samples. For mRNA and PUFA analyses, brain samples of the other hemisphere were flash frozen in liquid nitrogen and stored at −80 °C.

2.2.5.1. Neurotransmitters and their metabolites in the striatum, frontal cortex and hippocampus. Concentrations of DA, noradrenaline (NA), serotonin (5-HT), DA metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) and 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA), were measured in the supernatants by HPLC with electrochemical detector. HPLC analyses were conducted as described in detail in Ref. [27]. Data was expressed as pg per milligram of protein content.

2.2.5.2. Cytokine measurements in the striatum and midbrain. A customized 4-plex mouse cytokine panel (Biorad, CA, USA) consisting of fluorescent beads for pro-inflammatory cytokines interleukin-(IL)-1β, tumor necrosis factor-(TNF)-α and interferon-(IFN)-γ and anti-inflammatory cytokine interleukin-(IL)-10 was used with a Luminox protein suspension array system (Biorplex 200, Biorad, CA, USA), according to manufacturer’s instructions. This assay is highly sensitive and has been demonstrated to work well with detecting cytokines from brain tissues [27,35]. Data was expressed as pg per milligram of protein content.

2.2.5.3. Lipid extraction and gas chromatography. Frontal cortex tissue was analyzed with gas chromatography (GC). Lipids were extracted according to Folch’s method, as described in detail [10]. The fatty acid profiles were expressed as a percentage of the total microgram of fatty acid (weight percentage). Frontal cortex tissue was used as significant changes in PUFA content are observed in this tissue following dietary supplementation [17].

2.2.5.4. Real-time PCR of cFLAZ and COX-2. Brain tissue was lysed in TRI-reagent (Sigma, Canada) (1 ml per 50–100 mg tissue) by sonication (Misonix Inc., XL2000, NY, USA) at Power level 2. The RNA extraction method was followed according to manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the Quantscript reverse transcription kit (Qiagen, Canada) according to manufacturer’s instructions. Primer sequences for the target genes (Table 2) were obtained from NCBI’s Nucleotide database and designed in PRIME3 (Biology Workbench 3.2). Synthesis of these primers was performed by Sigma-Aldrich (Canada). Primers were tested for specificity in NCBI-nucleotide-BLAST. Polymerase chain reactions were carried out with Quantitect SYBR Green (Qiagen, Canada, 1) master mix kits, according to manufacturer’s instructions.

Table 1

<table>
<thead>
<tr>
<th>Standard chow fat contents (% of total mass)</th>
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<tbody>
<tr>
<td>Crude fat</td>
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<tr>
<td>Fatty acid contents in crude fat (% of crude fat)</td>
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<tr>
<td>C18:1 n-9 Oleic</td>
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<tr>
<td>C18:2 n-6 Linoleic</td>
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<tr>
<td>C18:3 n-6 Gamma Linolenic</td>
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<tr>
<td>C20:4 n-6 Arachidonic</td>
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<tr>
<td>C20:3 n-3 Eicosapentaenoic acid</td>
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<tr>
<td>C22:5 n-3 Docosapentaenoic acid</td>
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<tr>
<td>C22:6 n-3 Docosahexaenoic acid</td>
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<td>Total n-6</td>
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<td>Total n-3</td>
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<table>
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<tr>
<th>Experimental diet PUFA content (% of total mass)</th>
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<tr>
<td>Total n-6 content palm oil diet</td>
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<tr>
<td>Total n-3 content palm oil diet</td>
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<tr>
<td>Total EPA content palm oil diet</td>
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<tr>
<td>Total n-6 content E-PUFA diet</td>
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<td>Total n-3 content E-PUFA diet</td>
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<td>Total EPA content E-PUFA diet</td>
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<tr>
<td>N-6:3 ratio palm oil diet</td>
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<td>N-6:n-3 ratio E-PUFA diet</td>
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For preparation of the diets, the master bottle of E-PUFA (oil) was taken from −20 °C freezer and oil aliquoted in several 15 ml Falcon tubes that were flushed with nitrogen gas to slow oxidation. The appropriate volume of oil (0.8% in predetermined amount of chow) was then added to the chow and carefully mixed. For palm oil preparation, the palm oil had to be heated (50 °C) first to liquidize the fat. The prepared diets were fed immediately to the mice (10 g per mouse per day). The diets were fed daily and prepared each time for three days. The EPA diet was stored in −20 °C between feeding to further slow down oxidation. The palm oil diet was stored in 4 °C between feeding.

* Courtesy of LabDiet, PMI Nutrition International, LLC.
2.3. Statistics

Statistics were performed on GraphPad Prism 4.03 and SPSS 17.0 for Windows. Data were analyzed with two-way ANOVA, with main effects for factors MPTP-P, DIET and their interaction (MPTP-P × DIET). For body weight measurements and Morris water maze performance, a three-way repeated-measures ANOVA was performed, with between-subject factors DIET and MPTP-P; within-subject factors TIME (the weeks that body weight was sampled) or DAY (testing days in the Morris water maze), and their interactions. Bonferroni post hoc analysis was used to compare significance among the four groups in case of significant main effects. Bivariate correlations were calculated with the Pearson correlation coefficient r.

3. Results

3.1. Body weight

Body weight was significantly increased over time in all four groups \((\text{TIME}: F[16,518] = 88.9, P < 0.0001)\) as the mice were growing, but with progression of MPTP-P treatment (from day 8 of MPTP-P treatment and onwards), MPTP-P started to have a suppressing effect on body weight \((\text{MPTP-P} \times \text{TIME}: F[16,518] = 4.23, P < 0.0001)\), as evident in Fig. 2. Although MPTP-P only suppressed body weight in the palm oil treated mice, the E-EPA diet did not fully attenuate this effect, as the difference between MP and ME groups was not significant. The E-EPA diet by itself also did not affect body weight.

3.2. Behavior

3.2.1. Rotorod

A significant main effect of MPTP-P was found, but not of DIET and there was no interaction between MPTP-P and DIET. Nonetheless, MPTP-P significantly decreased the distance mice walked on the rotorod only in the palm oil group \((P-MP: t = 3.9, P < 0.05, \text{Bonferroni post hoc after two-way ANOVA})\) (Fig. 3A), suggesting that E-EPA partially attenuated the MPTP-P induced rotorod impairment.

3.2.2. Pole test

A significant main effect of MPTP-P was found, which significantly interacted with DIET \((\text{MPTP-P} \times \text{DIET}: F[1,34] = 4.2, P < 0.05)\). Bonferroni tests showed that MPTP-P significantly increased the total time which the mice needed to climb down the pole \((P-MP: t = 3.48, P < 0.01)\) and E-EPA attenuated this effect \((MP-ME: t = 2.72, P < 0.05)\) (Fig. 3B).

3.2.3. Open field

On line crosses, a significant main effect of MPTP-P was found, but not of DIET. MPTP-P significantly increased the line crosses (distance moved), but again only in the palm oil group \((P-MP: t = 2.77, P < 0.05)\). Bonferroni tests showed that E-EPA fed mice also increased line crosses and this effect bordered significance. In a former pilot study, we found that E-EPA significantly increased line crosses and rearings compared to palm oil. To determine whether this increase in the activity had a possible anxiety component, the percentage of time spent in the center area was measured. While MPTP-P did not have an effect on center exploration, there was a main effect of DIET. The percentage of time spent in the center area was increased by E-EPA feeding compared to palm oil in saline treated mice \((P-E: t = 2.61, P < 0.05)\) (Fig. 3D). There were no significant main or interaction effects on grooming and rearing \((\text{data not shown})\).

3.2.4. Morris water maze

No significant main effect of MPTP-P or DIET was found on latency to escape on the visual platform. However, there was an interaction between MPTP-P and DIET that approached significance \((F[1,31] = 3.12, P < 0.05)\), justifying post hoc tests. Bonferroni tests revealed that compared to saline treated mice, MPTP-P treated mice that were fed palm oil but not E-EPA had significantly longer escape latencies \((P-MP: t = 2.38, P < 0.05)\) (Fig. 4A) without a change in swimming speed \((\text{data not shown})\), suggesting that E-EPA partially attenuated the effect of MPTP-P. In the hidden platform version of the test, a learning curve over the three testing days can be seen in Fig. 3B, which was confirmed by the significant within-subject factor \((\text{DAY}: F[2,62] = 27.7, P < 0.0001)\) of the repeated measures analyses. Importantly, there was no interaction between DAY and the treatment factors MPTP-P or DIET, suggesting that all groups had a similar rate of learning over the three testing days (Fig. 4B). However, there was a significant between-subject effect of MPTP-P \((F[1,33] = 29.3, P < 0.0001)\), suggesting that the toxin did affect the average escape latency compared to saline. Multivariate ANOVA for each day showed that MPTP-P significantly increased escape latency on day 1 in palm-oil fed mice \((P-MP: t = 4.5, P < 0.01, \text{Bonferroni test})\) (Fig. 4C), but not significantly on day 2 and 3 \((\text{data not shown})\). No effect of MPTP-P or E-EPA was found on velocity \((\text{data not shown})\).

Probe trial performance is an important measure of acquired spatial memory. On total number of annulus crossings (Fig. 4D) or percentage of time spent in the correct quadrant, which indicate whether the subjects memorized, respectively, the exact and coarse location of the platform, there were no significant effects of MPTP-P or DIET. On the total number of quadrant entries however, there was a main effect of MPTP-P and it was found that MPTP-P significantly increased the total number of quadrant entries \((P-MP: t = 2.8, P < 0.05, \text{Bonferroni test after two-way ANOVA})\), which was reversed by E-EPA treatment \((MP-ME: t = 2.5, P < 0.05)\) (Fig. 4E).
Fig. 3. Hypokinesia and anxiety-related behavior. (A) Rotorod performance in the four groups, measured as the total distance moved (m), averaged over 5 consecutive trials. (B) Pole test total time (s) in the four groups, measured as the time the mouse needed to complete the test (inversely and climb down the pool), averaged over three consecutive trials. (C) Line crosses in the open field, a measure of locomotor activity, measured during the 5 min trial. (D) Anxiety in the open field, measured as the percentage of time spent in the center area, during the 5 min session. Data are presented as mean ± SEM. *P < 0.05 (P-MP); **P < 0.01 (P-MP); #P < 0.05 (MP-ME); P < 0.05 (P-E). P (palm oil + saline-probenecid); E (E-EPA + saline-probenecid); MP (palm oil + MPTP-probenecid); ME (E-EPA + MPTP-probenecid).

Fig. 4. Morris water maze: Performance in the Morris water maze in the four groups. (A) Escape latency (s) in the “cued version” (visual platform) of the test, averaged over four trials. (B) Learning curve of escape latency (s) over the three consecutive days of testing in the “spatial version” (hidden platform) of the test. Presented is the daily average of four trials. (C) Group comparison on day 1 of the “spatial version” of the test. (D) and (E) Performance on the “probe trial”, in which the platform was removed from the pool. (D) Group comparison on annulus crossings; the mean number times each mouse swam through the “annulus”, the exact area where the platform was located during the preceding spatial learning (hidden platform) trials. (E) The mean number of quadrant entries during the probe trial. Results are presented as mean ±SEM. *P < 0.05 (P-MP); **P < 0.01 (P-MP); *P < 0.05 (MP-ME). P (palm oil + saline-probenecid); MP (palm oil + MPTP-probenecid); ME (E-EPA + MPTP-probenecid).
On velocity in the probe trial, there were no significant effects of MPTP-P or DIET.

3.3. Neurochemistry

Results for neurochemistry in the striatum, frontal cortex and hippocampus are respectively shown in Tables 3–5.

3.3.1. Striatum

MPTP-P caused significant main effects on catecholamine DA and its metabolites DOPAC and HVA, but no interaction with DIET was found on all these measures. Indeed, the decrease of DA by MPTP-P was remarkably similar between diets; 79% in the palm oil (P-MP: P < 0.001, Bonferroni test after two-way ANOVA) and 81% in E-EPA group (E-ME: P < 0.001). Similarly, DOPAC was depleted 63% in the palm (P-MP: P < 0.001) and 62% in the E-EPA group (E-ME: P < 0.001), HVA was depleted 50% in the palm oil (P-MP: P < 0.01) and 52% in the E-EPA group (E-ME: P < 0.01). While HVA and DOPAC were decreased, DA was depleted more, so the ratios of DOPAC and HVA to DA were significantly increased. For both ratios, there was a significant main effect of MPTP-P, but again no interaction with DIET. Nonetheless, the DOPAC to DA was increased by 125% in the palm oil group (P-MP: P < 0.01), but only 40% in the E-EPA group (not significant), suggesting that the E-EPA diet did cause some modulation of the MPTP-P effect on DA turnover. However, the HVA/DA ratio was not different between diet groups, as it was increased, 250% in the palm (P-MP: P < 0.001) and 260% in the E-EPA group (E-ME: P < 0.001). Quite differently, however, on NA, there was a significant interaction between MPTP-P and the DIET factor (F[1,30] = 11.6, P = 0.0021). MPTP-P significantly decreased NA level by 58% in the palm oil group (P-MP: P < 0.01), while E-EPA feeding by itself (no MPTP-P treatment) also decreased NA level (P-E: P < 0.01), it significantly reversed the MPTP-P-induced depletion of NA (MP-ME: P < 0.05). There was also an interaction between MPTP-P and DIET factors on 5-HT metabolite 5-HIAA (F[1,29] = 3.9, P = 0.052). Post hoc tests indicated that MPTP-P increased 5-HIAA levels by 19% (P-MP: P < 0.05) and this effect was attenuated by E-EPA (MP-ME: P < 0.05).

3.3.2. Frontal cortex

Both MPTP-P and DIET caused main effects on DA levels, but there was no interaction. Post hoc Bonferroni testing indicated that MPTP-P decreased DA levels by 35% in the palm (P-MP: P < 0.05) and 40% in E-EPA fed mice (E-ME: P < 0.01). Interestingly, E-EPA treatment by itself increased frontal cortex DA levels by 43% compared to palm oil (P-E: P < 0.05). MPTP-P increased the DOPAC/DA ratio by 107% in E-EPA fed mice (E-ME: P < 0.05), compared to 33% in palm oil fed mice (not-significant). There was also a main effect of MPTP-P on NA and post hoc tests revealed that NA was depleted by 13% in E-EPA (E-ME: P < 0.001) versus 7% (not significant) in palm oil fed mice. Interestingly, the cortical 5-HIAA/5-HT ratio was 73% higher in MPTP-P treated E-EPA fed mice compared to MPTP-P treated palm oil fed mice (MP-ME: P < 0.05).

3.3.3. Hippocampus

In this brain region, MPTP-P, but not DIET caused a main effect on DA. MPTP-P depleted DA by 34% in the palm oil (P-MP: P < 0.05, Bonferroni test after two-way ANOVA) and 49% in E-EPA fed mice (E-ME: P < 0.01). On metabolite DOPAC, there was a significant interaction between MPTP-P and DIET (F[1,32] = 14.92, P < 0.001), justifying a post hoc test. MPTP-P increased DOPAC levels in the hippocampus by 116% in palm-oil fed mice (P-MP: P < 0.01). Although E-EPA by itself also increased DOPAC levels by 116% (P-E: P < 0.05), when combined with MPTP-P, E-EPA reduced DOPAC levels to those of palm oil control (saline) levels (MP-ME: P < 0.05). Due to depletion of DA in the hippocampus and increased levels of metabolite...
Table 4
Effect of MPTP/P and E-EPA treatment on neurotransmitter content in the frontal cortex.

<table>
<thead>
<tr>
<th>Neuro-transmitter</th>
<th>P Mean (SEM) N=9</th>
<th>E Mean (SEM) N=9</th>
<th>MP Mean (SEM) N=10</th>
<th>ME Mean (SEM) N=8</th>
<th>Bonferroni t-test (only significant mean differences) t, P&lt;0.05, 0.01, 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>8.6 (0.9)</td>
<td>12.3 (1.3)</td>
<td>5.6 (0.6)</td>
<td>7.4 (1.9)</td>
<td>2.6, P&lt;0.05, 2.5, P&lt;0.05, 3.1, P&lt;0.01</td>
</tr>
<tr>
<td>DOPAC</td>
<td>1.5 (0.2)</td>
<td>1.4 (0.2)</td>
<td>1.5 (0.1)</td>
<td>1.4 (0.1)</td>
<td></td>
</tr>
<tr>
<td>HVA</td>
<td>15.6 (0.9)</td>
<td>14.1 (1.7)</td>
<td>14.7 (1.6)</td>
<td>11.1 (1.4)</td>
<td></td>
</tr>
<tr>
<td>DOPAC/DA</td>
<td>0.18 (0.04)</td>
<td>0.13 (0.04)</td>
<td>0.24 (0.04)</td>
<td>0.27 (0.05)</td>
<td>2.9, P&lt;0.05, 2.5, P&lt;0.05, 3.1, P&lt;0.01</td>
</tr>
<tr>
<td>HVA/DA</td>
<td>1.83 (0.26)</td>
<td>1.44 (0.13)</td>
<td>2.37 (0.34)</td>
<td>2.40 (0.40)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>181.2 (4.0)</td>
<td>190.2 (3.2)</td>
<td>169.2 (4.1)</td>
<td>167.1 (3.8)</td>
<td>4.3, P&lt;0.001, 2.9, P&lt;0.05, 3.1, P&lt;0.01</td>
</tr>
<tr>
<td>5-HT</td>
<td>1.1 (0.2)</td>
<td>0.9 (0.2)</td>
<td>0.8 (0.1)</td>
<td>0.5 (0.07)</td>
<td></td>
</tr>
<tr>
<td>5-HIAA</td>
<td>6.1 (0.5)</td>
<td>6.9 (0.6)</td>
<td>7.2 (0.8)</td>
<td>7.3 (0.3)</td>
<td></td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>5.5 (0.66)</td>
<td>7.6 (0.87)</td>
<td>9.1 (1.5)</td>
<td>13.2 (1.3)</td>
<td>3.1, P&lt;0.05, 2.9, P&lt;0.05, 3.1, P&lt;0.01</td>
</tr>
</tbody>
</table>

Presented are the neurotransmitter concentrations (pg/mg protein) in the frontal cortex, for each treatment group. Results of Bonferroni t-tests are shown as well, in case significant omnibus tests were found. Alpha level was set at 0.05. For the Bonferroni tests, t and P-values are shown. CP (palm oil + saline-probenecid); CE (E-EPA + saline-probenecid); MP (palm oil + MPTP-probenecid); ME (E-EPA + MPTP-probenecid). DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DOPAC/DA, ratio of DOPAC to DA (indicator of DA turnover); HVA/DA, ratio of HVA to DA (indicator of DA turnover); NA, noradrenaline/norepinephrine; SHT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HIAA/5-HT, 5-HIAA to 5-HT ratio (indicator of 5-HT turnover).

Table 5
Effect of MPTP-P and E-EPA treatment on neurotransmitter content in the hippocampus.

<table>
<thead>
<tr>
<th>Neuro-transmitter</th>
<th>P Mean (SEM) N=9</th>
<th>E Mean (SEM) N=9</th>
<th>MP Mean (SEM) N=10</th>
<th>ME Mean (SEM) N=8</th>
<th>Bonferroni t-test (only significant mean differences) t, P&lt;0.05, 0.01, 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>2.4 (0.3)</td>
<td>2.9 (0.3)</td>
<td>1.6 (0.2)</td>
<td>1.5 (0.2)</td>
<td>2.8, P&lt;0.05, 2.5, P&lt;0.05, 4.1, P&lt;0.01</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.6 (0.05)</td>
<td>1.3 (0.2)</td>
<td>1.3 (0.2)</td>
<td>0.6 (0.2)</td>
<td></td>
</tr>
<tr>
<td>HVA</td>
<td>9.1 (1.5)</td>
<td>10.3 (1.4)</td>
<td>10.0 (1.5)</td>
<td>8.7 (1.8)</td>
<td></td>
</tr>
<tr>
<td>DOPAC/DA</td>
<td>0.30 (0.05)</td>
<td>0.46 (0.09)</td>
<td>0.82 (0.11)</td>
<td>0.30 (0.04)</td>
<td>4.3, P&lt;0.01, 3.8, P&lt;0.01, 2.8, P&lt;0.05</td>
</tr>
<tr>
<td>HVA/DA</td>
<td>4.07 (1.01)</td>
<td>3.6 (0.63)</td>
<td>6.2 (0.91)</td>
<td>4.8 (0.75)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>132(6.6)</td>
<td>129 (4.4)</td>
<td>135(5.2)</td>
<td>132 (5.2)</td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>3.0 (0.2)</td>
<td>2.5 (0.2)</td>
<td>2.1 (0.2)</td>
<td>2.8 (0.5)</td>
<td>2.8, P&lt;0.05, 2.5, P&lt;0.05, 3.1, P&lt;0.01</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5.4 (0.7)</td>
<td>4.2 (0.6)</td>
<td>4.8 (0.5)</td>
<td>5.1 (0.7)</td>
<td></td>
</tr>
<tr>
<td>5-HIAA/5-HT</td>
<td>2.5 (0.08)</td>
<td>2.6 (0.21)</td>
<td>3.2 (0.31)</td>
<td>2.6 (0.09)</td>
<td></td>
</tr>
</tbody>
</table>

Presented are the neurotransmitter concentrations (pg/mg protein) in the hippocampus, for each treatment group. Results of Bonferroni t-tests are shown as well, in case significant omnibus tests were found. Alpha level was set at 0.05. For the Bonferroni tests, t and P-values are shown. P (palm oil + saline-probenecid); E (E-EPA + saline-probenecid); MP (palm oil + MPTP-probenecid); ME (E-EPA + MPTP-probenecid). DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DOPAC/DA, ratio of DOPAC to DA (indicator of DA turnover); HVA/DA, ratio of HVA to DA (indicator of DA turnover); NA, noradrenaline/norepinephrine; SHT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HIAA/5-HT, 5-HIAA to 5-HT ratio (indicator of 5-HT turnover).
DOPAC, there was a main effect of MPTP-P on DOPAC/DA levels and this effect interacted with DIET (MPTP-P × DIET: F(1,32) = 14.02, P < 0.001). MPTP-P significantly increased the DOPAC/DA ratio by 166% (P < 0.01) and E- EPA treatment attenuated this effect (MP-ME: P < 0.01). On 5-HT, an interaction effect between BBR-7318 and DIET factors (F(1,28) = 5.07, P < 0.05) was found. Post hoc analyses indicated that MPTP-P significantly decreased hippocampal 5-HT by 30% (P-MP: P < 0.05), but only in the palm oil group.

3.4. Cortex n-3 and n-6 fatty acid contents

MPTP-P did not affect the concentration of EPA, docosapentaenoic acid (DPA) and DHA in cortical tissue, but EPA feeding by itself had significant main effects on EPA and DPA. The post hoc tests showed that the E- EPA diet significantly increased the concentrations of EPA in both saline (P-E: t = 8.593, P < 0.001) and MPTP-P treated mice (MP-ME: t = 9.691, P < 0.001) (Fig. 5A). Likewise, DPA was significantly increased by EPA feeding in both saline (P-E: t = 8.607, P < 0.001) and MPTP-P treated mice (MP-ME: t = 7.289, P < 0.001) (Fig. 5B). On the other hand, the E- EPA diet did not affect DHA concentration (Fig. 5C) or n-3 fatty acid α- LA (data not shown). With respect to n-6 fatty acids, neither MPTP-P nor the DIET factor had any effect on the tissue content of the major n-6 fatty acids linoleic acid (LA), gamma-linolenic acid (GLA) and AA (data not shown).

3.5. Cytokine concentrations in the striatal and midbrain

In the striatum, there were significant main effects of MPTP-P on TNF-α and INF-γ concentrations. The effect of MPTP-P on TNF-α levels interacted significantly with DIET [MPTP-P × DIET: F(1,32) = 3.92, P < 0.05]. Indeed, post hoc tests showed that MPTP-P increased TNF-α expression compared to saline in the palm oil group (P-MP: t = 2.93, P < 0.05), and this effect was attenuated by E- EPA feeding (MP-ME: t = 2.4, P < 0.05) (Fig. 6A). Post hoc analyses also indicated that MPTP-P significantly increased INF-γ levels compared to saline, and this effect was only found significant in the palm oil group (P-MP: t = 2.47, P < 0.05) (Fig. 6B), suggesting that E- EPA partially attenuated the effect of MPTP-P. No significant effects were detected on IL-1β and anti-inflammatory cytokine IL-10 (data not shown). In the midbrain, MPTP-P as well as DIET caused a significant main effect on anti-inflammatory cytokine IL-10. Post hoc analyses indicated that MPTP-P increased IL-10 in the palm oil group (P-MP: t = 2.4, P < 0.05) and this effect was attenuated by E- EPA feeding (MP-ME: t = 2.7, P < 0.05) (Fig. 5C).

3.6. Striatal and midbrain mRNA expression of COX-2 and cPLA2

In the striatum, there was a significant interaction between MPTP-P and DIET on COX-2 mRNA expression (MPTP-P × DIET: F(1,15) = 11.34, P < 0.01). The post hoc analyses showed that COX-2 mRNA expression was significantly downregulated by MPTP-P compared to saline in the palm oil group (P-MP: t = 3.15, P < 0.05) (Fig. 7A). E- EPA feeding by itself also significantly downregulated COX-2 mRNA expression compared to palm oil in saline treated mice (P: t = 2.6, P < 0.05) (Fig. 7A). There were no significant effects of MPTP-P or DIET on cPLA2 mRNA expression (Fig. 7B). In the midbrain, no significant effects of either MPTP-P or E- EPA treatment on cPLA2 and COX-2 mRNA expression were found (data not shown).

4. Discussion

For the first time, we tested whether E- EPA can protect against experimental PD induced by MPTP-P. The E- EPA diet significantly increased brain levels of EPA and DPA, attenuated or improved the hypokinesia and procedural memory impairment induced by MPTP-P, and prevented an increase in striatal pro-inflammatory cytokines. While E- EPA did attenuate some alterations in striatal and hippocampal neurochemistry, there was no clear pattern of protection at neurochemical level. Furthermore, the E- EPA diet did not attenuate the loss of nigrostriatal DA, which is a major aspect of PD neuropathology. With this mixed data, combined with the protective effects of n-3 PUFA reported in other studies, we suggest that E- EPA may have potential as a protectant in PD, but further research is warranted.

4.1. Behavior and neurochemistry

Consistent with PD hypokinesia, MPTP-P presently caused an impairment of pole and rotorod performance, but also open field hyperactivity. This MPTP-induced hyper- rather than hypo-activity in spontaneous movement paradigms, such as the open field, has been described frequently, especially when more chronic and high accumulative dose regimens are used [27,36,37]. This may be a form of hyperactive disinhibition, caused by a fronto-striatal hypodopaminergic state [38]. It was probably not anxiety related, as open field centre square exploration was not different from controls. Other studies also did not report MPTP effects on mood [43]. The increase in quadrant entries, indicating an increase in swimming activity in MPTP-P treated mice, as observed in the water maze, may be similar disinhibition. There was presently little evidence that MPTP-P treated mice had a spatial memory impairment. Only a few studies have assessed spatial memory in MPTP treated mice and while a spatial memory impairment was observed in one (which used another MPTP-regimen) [39], these results concurred with others studies demonstrating only an impairment in the cued or working memory version of the test [33,40]. The visual (cued) platform test relies on basal ganglia rather than hippocampal integrity [33,34]. It is unclear why MPTP-P treated mice had longer escape latencies on the first day of hidden platform testing. This may be a working memory impairment, as mice were trying to learn the new task, while attempting to memorize the location of the platform. Swimming was not impaired in MPTP-P treated mice and there was no change in velocity.

Consistent with PD neuropathology [1], the most robust effects of MPTP-P on neurochemistry were found in the striatum. Presently, MPTP-P caused a massive depletion of DA, while the ratios of DOPAC and HVA to DA increased, as more DA was released and metabolized from remaining nigrostriatal terminals, a compensatory mechanism observed in PD [51] [41]. As in PD [1,41], MPTP-P also caused changes in striatal non-dopaminergic neurochemistry. Similar to our previous findings with this model [27], 5-HT metabolite levels were increased. Since MPP+ has less affinity to the 5-HT transporter, this effect may be an adaptation or compensation in striatal serotonergic neurochemistry in response to MPTP-P rather than a direct effect of the neurotoxin [1,41,42]. These potentially adaptive neurochemical changes may be behaviorally relevant, as pole test and rotorod performance in the MPTP-P palm oil group were correlated to striatal DA (R = 0.638, P < 0.05) and 5-HT metabolism (R = −0.669, P < 0.05) respectively.

Further consistent with PD [1,41], MPTP-P caused significant extra-striatal neuropathology. While a depletion of cortical DA is a common finding with MPTP-P [27] and other MPTP regimens [37], a new finding was the abnormalities in hippocampal neurochemistry. While the hippocampus is generally ignored in MPTP research, these findings may be consistent with hippocampal neuropathology observed in some PD patients [5]. Yet the behavioral relevance of this effect is unclear. Long-term potentiation (LTP), a neurophysiological process during learning and memory, has been linked to dopaminergic neurochemistry in the hippocampus [44], however, memory performance was presently not affected by MPTP-P. Furthermore, we recently (data submitted) found that
MPP+ administration to hippocampal brain slices does not affect LTP, despite a significant decrease in DA content.

4.2. Brain PUFA, and inflammation

Neuroinflammation is suggested to be an important aspect of PD pathogenesis [6]. Consistent with cytokine production and microglia activation in PD and the MPTP-P model [27,45], MPTP-P presently increased TNF-α and IFN-γ in the striatum. Knock-out of both these major pro-inflammatory cytokines or receptors in mice causes resistance to MPTP neurotoxicity [46,47]. The present increase in anti-inflammatory cytokine IL-10 in the midbrain, which was documented before (in the striatum) in another MPTP regimen [48], may be a compensatory mechanism against the nigral

Fig. 5. Brain n-3 PUFA concentrations: Levels of brain fatty acids in the four treatment groups, measured from frontal cortex extracts with GC. The fatty acid profiles were expressed as a percentage of the total microgram of fatty acid (weight percentage). (A) Eicosapentaenoic acid (C20:5, n-3); (B) Docosapentaenoic acid (C22:5, n-3); (C) Docosahexaenoic acid (C22:6, n-3). Data are presented as mean ± SEM. ***P < 0.001 (E-P); ###P < 0.001 (ME-MP). P (palm oil + saline-probenecid); E (E-EPA + saline-probenecid); MP (palm oil + MPTP-probenecid); ME (E-EPA + MPTP-probenecid).

Fig. 6. Cytokine levels: Striatal and midbrain cytokines in the four treatment groups, measured with the Bioplex protein suspension array system from striatal and midbrain extracts. Data was expressed as pg per milligram of protein content. (A) Striatal pro-inflammatory cytokine TNF-α; (B) Striatal pro-inflammatory and Th-1 cytokine IFN-γ; (C) Midbrain anti-inflammatory cytokine IL-10. Data are presented as mean ± SEM. *P < 0.05 (P-MP); #P < 0.05 (ME-MP). P (palm oil + saline-probenecid); MP (palm oil + MPTP-probenecid); ME (E-EPA + MPTP-probenecid).

Fig. 7. cPLA2 and COX-2 mRNA expression. Striatal COX-2 and cPLA2 mRNA expression, measured with real-time (q)-PCR from striatal mRNA extracts. Presented are the fold changes of target gene expression relative to reference gene (beta-actin) expression, calculated with the ΔΔCt correction. (A) Cyclo-oxygenase-2 (COX-2); (B) Cytosolic phospholipase A2 (cPLA2). Data are presented as mean ± SEM. *P < 0.05 (P-MP); #P < 0.05 (P-E). P (palm oil + saline-probenecid); E (E-EPA + saline-probenecid); MP (palm oil + MPTP-probenecid).
prevented increases of striatal TNF-α and partially attenuated the increase in IFN-γ, a potent microglia activator. These effects of E-EPA may have neuroprotective relevance, since TNF-α and IFN-γ knock-out mice are resistant to MPTP neurotoxicity [46,47]. Anti-inflammatory treatments have protective effects in PD and experimental PD [51], but unlike E-EPA, they have side effects [52]. These anti-inflammatory effects of E-EPA may also explain that it prevented MPTP-P induced weight loss, as these cytokines can have anorexigenic effects [53].

Altogether, while the present evidence in support of neuroprotective effects of E-EPA in experimental PD is limited by the lack of nigrostriatal protection, it does demonstrate that E-EPA, despite its low content in the brain, is a neuroactive lipid that may have some beneficial effect in PD. Further research with different concentrations of E-EPA, different diet times and different dosing regimens of the MPTP model may provide further insights.

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