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Molecular and Cellular Pharmacology

Ethyl-eicosapentaenoate modulates changes in neurochemistry and brain lipids induced by parkinsonian neurotoxin 1-methyl-4-phenylpyridinium in mouse brain slices

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ABSTRACT

Evidence suggests a link between Parkinson's disease and the dietary intake of omega (n)–3 and n–6 polyunsaturated fatty acids (PUFAs). Presently, we investigated whether an acute dose of parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) affects brain n–3 and n–6 PUFA content and expression of fatty acid metabolic enzymes cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2) in brain slices from C57Bl/6 mice. Furthermore, we investigated whether feeding a diet of n–3 PUFA ethyl-eicosapentaenoate (E-EPA) to these mice can attenuate the MPP⁺ induced changes in brain PUFA content and expression of cPLA2 and COX-2, and attenuate MPP⁺ induced changes in neurotransmitters and metabolites and apoptotic markers, bax, bcl-2 and caspase-3. MPP⁺ increased brain content of n–6 PUFAs linoleic acid and arachidonic acid, and increased the mRNA expression of cPLA2. MPP⁺ also depleted striatal dopamine levels and increased dopamine turnover, and depleted noradrenaline levels in the frontal cortex. The neurotoxin induced increases in bax, bcl-2 and caspase-3 mRNA expression that approached significance. E-EPA by itself increased brain n–3 content, including EPA and docosapentaenoic acid (C22:5, n–3), and increased cortical dopamine. More importantly, E-EPA attenuated the MPP⁺ induced increase in n–6 fatty acids content, partially attenuated the striatal dopaminergic turnover, and prevented the increases of pro-apoptotic bax and caspase-3 mRNAs. In conclusion, increases in n–6 PUFAs in the acute stage of exposure to parkinsonian neurotoxins may promote pro-inflammatory conditions. EPA may provide modest beneficial effects in Parkinson's disease, but further investigation is warranted.

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1. Introduction

Parkinson's disease is a neurodegenerative disease, characterized by apoptosis and degeneration of nigrostriatal dopaminergic neurons and additional degeneration in the frontal cortex and noradrenergic neurotransmitter system (Dauer and Przedborski, 2003; Rabey and Burns, 2002). While the cause for Parkinson's disease is still unclear, recent findings have indicated that inflammation may be involved in

the onset or progress of Parkinson's disease (Dauer and Przedborski, 2003). In relation to this, increasing evidence suggests an important role of omega-(n)–3 and omega-(n)–6 polyunsaturated fatty acids (PUFAs) in the pathology and therapy of Parkinson's disease and other neurodegenerative disorders, such as Alzheimer's disease (AD). PUFAs enhance the fluidity of neuronal membranes, thereby modulating vital functions such as vesicular storage and release of neurotransmitters (Youdim et al., 2000). Furthermore, PUFAs, such as n–6 fatty acid arachidonic acid and n–3 fatty acid eicosapentaenoic acid (EPA) can be converted to highly neuroactive molecules called eicosanoids by enzymatic action of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2). Although eicosanoids are important for neuronal signaling, they may have pro-inflammatory effects when derived from arachidonic acid, whereas those derived from EPA generally have anti-inflammatory action (Simopoulos, 2008; Russo, 2009). Furthermore, n–3 fatty acids are potent activators of peroxisome proliferator-activated receptors (PPARs), which inhibit the transcription of inflammatory mediators, including cytokines, cPLA2 and COX-2 (Bernardo and Minghetti, 2006; Lappas and Rice, 2004; Kitajka et al., 2002). Thus, not surprisingly, increased nutritional intake of n–6 fatty acids increases risk of inflammatory disorders and

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neurodegenerative disease, such as Parkinson's disease (Miyake et al., 2010), while protective effects of n-3 PUFA supplementation have been demonstrated in experimental animal models of Parkinson's disease (Delattre et al., 2010; Bousquet et al., 2008, 2009).

However, despite this apparent relation between intake of PUFAs and Parkinson's disease, these studies report no changes in brain fatty acid profiles in patients or experimental animal models of Parkinson's disease (Bousquet et al., 2008; Julien et al., 2006). It is possible that in the chronic stage of the disease, fatty acid metabolic pathways have stabilized and that most changes in fatty acid composition of neuronal membranes occur temporary during early disease development. An initial rise in n-6 fatty acids could contribute to the development of brain inflammation. Thus, the first objective of the present study was to test the hypothesis that an acute dose of 1-methyl-4-phenylpyridinium (MPP⁺), the active metabolite of parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), administered to mouse brain slices increases n-6 and decreases n-3 PUFAs while increasing the expression of cPLA2 and COX-2.

Previous studies investigating the neuroprotective effects of n-3 fatty acids in experimental models of Parkinson's disease mainly used fish oil containing the long chain n-3 PUFA docosahexaenoic acid (DHA), but less EPA. Although EPA is far less abundant in the brain than DHA, it is highly neuroactive (Horrobin, 2002), has more potent anti-inflammatory effects (Mickleborough et al., 2009; Sierra et al., 2008) and is more effective than DHA in the treatment of psychiatric disorders (Peet and Stokes, 2005). Therefore, presently, the second objective was to test the hypothesis that EPA supplementation to mice can reverse the effects of acute MPP⁺ and that these effects are mediated by alteration of brain EPA levels. To investigate both hypotheses of the present study, brain slices containing the striatum and frontal cortex were collected from EPA fed C57Bl/6 mice and briefly exposed to MPP⁺, followed by measurement of neurotransmitters and metabolites, brain fatty acid profiles and mRNA expression of cPLA2 and COX-2 and apoptotic markers, bax, bcl-2 and caspase-3.

2. Material and methods

2.1. Animals

All experimental procedures were carried out in agreement with the Canadian Council on Animal Care (CCAC) at the University of Prince Edward Island (UPEI). All experimental procedures were approved by the local animal care committee of UPEI. Forty male C57Bl/6 mice (Charles River, Canada), 10 weeks of age (25 g), were habituated for 1 week to the animal colony and had free access to food and water at all times. Animals were housed individually in a 12–12 light–dark cycle with room temperature at $\pm 21^\circ\text{C}$.

2.2. Diet

Mice were fed a diet consisting of regular chow (PMI[®] Richmond, Indiana, US) supplemented with highly purified 0.8% ethyl-eicosapentaenoate (E-EPA) (Amarin Neuroscience Ltd, UK) ($N=20$), or 0.8% palm oil (Sigma, Canada) ($N=20$). The nutritional composition of the diets is shown in Table 1. Palm oil was chosen to be the 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 (Song et al., 2008). Food was freshly prepared every three days, fed daily and stored in -20°C overnight. The feeding period was 6 weeks.

2.3. Chemicals

MPP⁺ iodide (Sigma, Canada, #D048) was dissolved in sterile dd H₂O aliquoted into stocks of 10 mM, which were frozen in -80°C . The selected concentration of 20 μM MPP⁺ and incubation time of 4 h as

Table 1

Fatty acid compositions diet.

Standard chow fat contents (% of total mass) ^a	
Crude fat	4.5
Fatty acid contents in crude fat (% of crude fat)	
C18:1 oleic	27.6
C18:2, n-6 linoleic	28.9
C18:3, n-6 gamma linolenic	<0.1
C18:3, n-3 linolenic	2.31
C20:4, n-6 arachidonic	0.30
C20:5, n-3 eicosapentaenoic	0.85
C22:5, n-3 docosapentaenoic	0.19
C22:6, n-3 docosahexaenoic	0.96
Total n-6	29.7
Total n-3	4.54
Experimental diet PUFA content (% of total mass)	
Total n-6 content palm oil diet	1.4
Total n-3 content palm oil diet	0.2
Total EPA content palm oil diet	0.04
Total n-6 content E-EPA diet	1.34
Total n-3 content E-EPA diet	1
Total EPA content E-EPA diet	0.84
N-6:n-3 ratio palm oil diet	7:1
N-6:n-3 ratio E-EPA diet	1.3:1

For preparation of the diets, the master bottle of EPA (oil) was taken from -20°C freezer and oil aliquoted in several 15 ml Falcon tubes that were flushed with nitrogen gas. The appropriate volume of oil (0.8% in pre-determined 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 slow down oxidation. The palm oil diet was stored in 4°C between feeding.

^a Courtesy of LabDiet, PMI Nutrition International, LLC.

an acute model were pre-determined from previous reports and a pilot experiment in which 20, 50 and 100 μM MPP⁺ were tested on striatal dopamine level for 2 and 4 h. Striatal dopamine was chosen as the main reference for choosing the concentration and incubation time of MPP⁺, since striatal dopamine is the primary target of MPP⁺ (Dauer and Przedborski, 2003). From the preliminary HPLC analyses (data not shown), it appeared that similar depletions of striatal dopamine were observed as in other studies at similar concentrations and incubation times. Similar to our findings, Ambrosio et al. (1996) found a bottom effect of MPP⁺ concentration on dopamine levels, with the largest decline of striatal dopamine level between 0 and 25 μM (2 h incubation) and no substantial further decline beyond this concentration. Since gene expression may be more expressed at 4 h MPP⁺ treatments rather than 2 h (Pattarini et al., 2007), a 4 hour incubation time was presently chosen.

2.4. Sacrifice and slice preparation

The brain slice model has been successfully applied to investigate the acute effects of parkinsonian neurotoxins (Ambrosio et al., 1996; Wilson et al., 1990; Snape et al., 1988). Following the diet, the animals were anesthetized with halothane and decapitated. Their brains were rapidly removed and placed in ice-cold, oxygenated bicarbonate-buffered (95% O₂:5% CO₂) artificial cerebral spinal fluid (aCSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26 NaHCO₃, and 10 glucose. A 0.5 cm² block of brain tissue was glued to a cutting surface, hemisected and (200–400 μM) coronal slices of each hemisection cut, using a Leica VT1000S microtome (Leica Microsystems). Brain slices were left to equilibrate in aCSF for approximately 1 h in a recovery chamber. The slices were then transferred to 6-well plates that contained oxygenated bicarbonate-buffered aCSF, with or without MPP⁺. From each animal, brain slices of one hemisection were treated with MPP⁺, whereas neuroanatomically matched brain slices of the other hemisection served as the untreated control (left and right hemispheres equally distributed between conditions). This experimental design ensured stable

baseline neurotransmitter levels between control and MPP⁺ treated brain slices. The aCSF was held at room temperature ($21 \pm 1^\circ\text{C}$), as it was suspected that 37°C may promote the degradation of tissue after subtle damage inflicted by the microtome. Following treatment, brain slices were washed twice in oxygenated bicarbonate-buffered phosphate buffered saline (PBS) and brain regions (striatum and frontal cortex) dissected rapidly on ice. Half of the animals ($N=20$) were used for HPLC analyses, and the other half ($N=20$) for fatty acid and gene expression analyses.

2.5. High performance liquid chromatography (HPLC)

Dissected brain tissue was sonicated (Misonix Inc, XL2000, NY, USA, Power level 2) in ice-cold buffer containing (1 L): L-ascorbic acid (8.8 mg); 70% HClO₄ (9.3 ml); ethylenediaminetetraacetic acid (EDTA) (100 mg), and centrifuged at 9000 g for 25 min at 4°C . Determination of neurotransmitters dopamine, noradrenaline, and dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) was performed using a BASi HPLC system (Analytical Systems, LLC, MN, USA). The column used was a Waters Symmetry Shield RP18 $3.5\ \mu\text{m}$, $4.6 \times 75\ \text{mm}$. The mobile phase consisted of (1 L): sodium acetate (6 g), EDTA (10 mg), sodium n-octyl sulfate (SOS) (100–200 mg) in 10% methanol. Acetic acid was added to reach a pH of 3.5. The results (analyzed with BASi Chromatograph) were expressed as pg per milligram of protein. Striatum samples were run twice; one time with a 100× dilution due to the high concentration of dopamine and one time without dilution (for dopamine metabolites and noradrenaline concentrations). For protein content analyses, sonicated brain tissue samples were centrifuged at 9000 g for 10 min at 4°C . Sample protein concentration from sonicates was measured with standard BCA protein assay (Pierce/Thermo Scientific, USA).

2.6. Lipid extraction and gas chromatography (GC)

Due to the sparse tissue available from brain slices, a combination of frontal cortex and striatal tissue was used for lipid analyses. The tissue was flash frozen in liquid nitrogen and stored in -80°C . Lipids were extracted according to Folch's method (Folch et al., 1957). C13:0 (Nu-Chek-Prep, N-13-A) was added as an internal standard. Brain tissue was homogenized with 5 ml chloroform/methanol solution (2/1). After agitating for 60 min at room temperature, 2 ml 1% NaCl was added and the mixture was centrifuged at 4000 g, to separate the two phases. The lower chloroform phase containing lipids was dried under a nitrogen stream and dried fatty acid samples incubated in a water bath at 80°C with 5 ml ethanolic KOH. After cooling, 2.5 ml 1% NaCl and 1.5 ml Hexane were added. After vortexing and phase separation, the top layer was discarded. HCl solution (35%) was added to adjust the pH to 2–2.5, followed by 2 ml hexane. After the separation, the top layer was dried under a nitrogen stream. Methanolic HCl (1.5 ml 3 N) was added to dry fatty acids, and incubated at 95°C for 60 min. After cooling, 2.5 ml 1% NaCl and 1.5 Hexane were added. After vortexing and phase separation, the top layer containing FAME (fatty acid methyl ester) was collected for GC analysis. FAME was analyzed by GC using a Hewlett Packard (Palo Alto, CA) 6890 equipped with a flame ionization detector. The FAME was separated on a 60 m Agilent 122-2361 DB-23 capillary column (0.25 mm diameter and 0.15 μm coating thickness) using helium at a flow rate of 1.8 ml/min. The chromatographic run parameters included a starting temperature of 50°C , increased at $25^\circ\text{C}/\text{min}$ to 175°C and at $10^\circ\text{C}/\text{min}$ to 225°C , where it was held for 17 min. The inlet and detector temperatures were constant at 225°C . Peaks were identified by comparison of retention times with external FAME standard mixtures (Supelco, 47885-U) and PUFA standard (Nu-Chek-Prep, 462). The fatty acid profiles were expressed as a percentage of the total microgram of fatty acid (weight percentage).

2.7. Extraction of total RNA, cDNA synthesis and quantitative real-time PCR

Striatal brain tissue was flash frozen in liquid nitrogen and stored in -80°C . The tissue was lysed in TRI-reagent (Sigma, Canada, #93289) (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 Quantiscript reverse transcription kit (Qiagen, Canada, #205310) according to manufacturer's instructions. Any possible gDNA contamination was removed in a quick DNAase step (42°C for 2 min). The polymerase and RNase-H reaction was realized by incubation at 42°C for 15 min followed by a de-activation step of 3 min at 95°C . The finished reaction could be used for real-time PCR. Primer sequences for the target genes (Table 2) were obtained from NCBI's Nucleotide database and designed in PRIMER3 (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, #204141) master mix kits, according to manufacturer's instructions. The PCR sequence was run on a Corbett Life Science (Sydney, Australia) Rotor-Gene 6000 system, and consisted of an incubation for 15 min at 95°C to activate the HotStarTaq DNA polymerase, followed by 94°C for 15 s (denaturing), 59°C for 30 s (annealing) and 72°C for 30 s (extension). After 40 cycles, a melting curve was generated for determination of primer specificity and identity. Gene expression levels were quantified by normalizing Ct values of target genes to Ct values of the reference gene (beta actin) with the $\Delta\Delta\text{Ct}$ method.

2.8. Statistics

Statistics were performed with Graphpad Prism 4.03 (San Diego, CA, USA) for Windows. Since data were analyzed as diet (E-EPA, or palm oil) by MPP⁺ intoxication, 2-way analyses of variance (ANOVA) was performed with main effects for factors DIET and MPP⁺, and their interactions (MPP⁺ × DIET). Significance was pre-determined to be at the 0.05 level. In case of significant main or interaction effects, post-hoc Bonferroni *t*-tests were done to compare means of the following four equally sized groups ($N=10$): P (palm oil, no MPP⁺ treatment); E (E-EPA, no MPP⁺ treatment); MP (palm oil, MPP⁺ treatment) and ME (E-EPA, MPP⁺ treatment).

3. Results

3.1. Striatal neurotransmitters and metabolites

Two-way ANOVA of HPLC data indicated that there was a significant effect of MPP⁺ but not DIET on striatal dopamine level ($F[1,32]=31.40$, $P<0.0001$) level. Post-hoc analyses showed that

Table 2
Primer names, sequences and product sizes (mouse source).

Primer name	Sequence	Product size (bp)
Actin F	TGTTACCAACTGGGACGACA	139
Actin R	CTGGGTTCATCTTTTCACGGT	
cPLA2 F	GACAGCTCCGACAGTGATGA	227
cPLA2 R	GACCAACTTGCTTGTTGT	
COX 2 F	CCAGATGCTATCTTTGGGGA	105
COX 2 R	GCTCGGCTCCAGTATTGAG	
BAX F	CTGGAAGAAGATGGGCTGAG	173
BAX R	GAAAAATGCCTTCCCTTC	
BCL 2 F	TGGCTGTCTCTGAAGACCTT	173
BCL 2 R	CTCACTTGTGGCCAGGTAT	
Caspase-3 F	GGAATTCGAGTCTTCTCCC	271
Caspase-3 R	AACAAACAGAAACACGCC	

Table 3Effect of MPP⁺ and EPA treatment on neurotransmitter content in the striatum.

Neurotransmitter	P Mean (S.E.M.)	E Mean (S.E.M.)	MP Mean (S.E.M.)	ME Mean (S.E.M.)	Bonferroni <i>t</i> -test (only significant results) <i>t</i> , <i>P</i> <0.05; <i>P</i> <0.01; <i>P</i> <0.001			
					P versus E	P versus MP	E versus ME	MP versus ME
DA	100.01 (0.06)	100.01 (0.07)	74 (0.07)	75 (0.05)			4.2, <i>P</i> <0.001	3.7, <i>P</i> <0.01
DOPAC	11.24 (0.89)	10.92 (1.39)	8.78 (1.05)	10.23 (1.76)				
DOPAC/DA	0.11 (0.09)	0.11 (0.013)	0.11 (0.013)	0.10 (0.013)				
HVA	6.6 (0.67)	6.6 (0.54)	7.6 (0.89)	6.03 (1.05)				
HVA/DA	0.61 (0.07)	0.59 (0.06)	0.94 (0.06)	0.67 (0.09)		3.1, <i>P</i> <0.05		
NA	7.4 (0.6)	6.7 (1.00)	6.14 (0.67)	6.28 (0.52)				

Neurotransmitter concentrations are expressed as pg/mg protein for the striatum, 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 + aCSF); E (E-EPA + aCSF); MP (palm oil + aCSF – MPP⁺); ME (E-EPA + aCSF – MPP⁺). 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.

MPP⁺ significantly decreased striatal dopamine level compared to control condition in both palm (P versus MP: *P*<0.001) and E-EPA (E versus ME: *P*<0.01) groups (Table 3). However, metabolite DOPAC, and the DOPAC to dopamine ratio were not affected by MPP⁺ (Table 3). On the other hand, there was a trend that MPP⁺ affected HVA level ($F[1,32]=5.2$, *P*=0.066), and the HVA to dopamine ratio was significantly altered ($F[1,32]=6.9$, *P*<0.05), while DIET did not affect this ratio. Post-hoc tests showed that MPP⁺ significantly increased the HVA to dopamine ratio compared to controls in the palm oil group (P versus MP: *P*<0.05) (Table 3), but not in the EPA group. Catecholamine noradrenaline was not affected by either MPP⁺ or DIET (Table 3).

3.2. Frontal cortex neurotransmitters and metabolites

There was no main effect of MPP⁺, but DIET significantly affected dopamine level in the frontal cortex ($F[1,32]=4.9$, *P*<0.05). Post-hoc tests indicated that E-EPA by itself significantly increased dopamine level compared to palm oil (P versus E: *P*<0.05) (Table 4). After MPP⁺ treatment, the dopamine concentration in E-EPA fed mice was similar to the value of the group fed palm oil, so the difference in dopamine levels between the E-EPA control group (E) and the E-EPA with MPP⁺ group (ME) was significant. However, since dopamine levels in E-EPA and palm oil groups did not differ after MPP⁺ treatment, there was no interaction between MPP⁺ and E-EPA treatments (Table 4). There were no significant main or interaction effects of MPP⁺ and DIET on DOPAC or the DOPAC to dopamine ratio in this brain region (Table 4). The HVA level was too low to be detected by the HPLC equipment. There was a significant effect of MPP⁺ on the noradrenaline level ($F[1,32]=19.39$, *P*<0.01), but DIET did not affect this neurotransmitter. The noradrenaline concentration was significantly decreased by MPP⁺ compared to controls in the palm oil (P versus MP: *P*<0.01) and to a lesser extent in the E-EPA group (E versus ME: *P*<0.05) (Table 4).

Table 4Effect of MPP⁺ and EPA treatment on neurotransmitter content in the frontal cortex.

Neurotransmitter	P Mean (S.E.M.)	E Mean (S.E.M.)	MP Mean (S.E.M.)	ME Mean (S.E.M.)	Bonferroni <i>t</i> -test (only significant results) <i>t</i> , <i>P</i> <0.05; <i>P</i> <0.01			
					P versus E	P versus MP	E versus ME	MP versus ME
DA	16.72 (1.46)	31.99 (7.72)	15.20 (3.12)	16.35 (3.98)	2.6, <i>P</i> <0.05		2.4, <i>P</i> <0.05	
DOPAC	2.06 (0.33)	2.5 (0.58)	1.74 (0.34)	1.91 (0.19)				
DOPAC/DA	0.11 (0.031)	0.06 (0.015)	0.10 (0.01)	0.13 (0.027)				
NA	106.4 (12.4)	108.5 (5.91)	59.74 (10.18)	74.74 (6.79)		3.6, <i>P</i> <0.01	2.6, <i>P</i> <0.05	

Neurotransmitter concentrations are expressed as pg/mg protein for 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. P (palm oil + aCSF); E (E-EPA + aCSF); MP (palm oil + aCSF – MPP⁺); ME (E-EPA + aCSF – MPP⁺). DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, DOPAC/DA, ratio of DOPAC to DA (indicator of DA turnover); NA, noradrenaline/norepinephrine.

3.3. Omega-3 fatty acid profiles in brain tissue

As suggested by two-way ANOVA of GC data, alpha-linolenic acid (C18:3, n–3) was not affected by either MPP⁺ or DIET (Fig. 1A). The concentration of eicosapentaenoic acid (EPA, C20:5, n–3) was also not affected by MPP⁺, but DIET significantly altered the EPA content ($F[1,33]=98.69$, *P*<0.001). Indeed, the E-EPA diet by itself (no MPP⁺ treatment) (P versus E: *P*<0.001) and after MPP⁺ treatment (MP versus ME: *P*<0.001), significantly increased tissue EPA content (Fig. 1B). Similarly, the docosapentaenoic acid (DPA, C22:5, n–3) content was not affected by MPP⁺, but DIET significantly altered the DPA content ($F[1,34]=28.90$, *P*<0.001). E-EPA significantly increased the DPA content by itself (P versus E: *P*<0.001) and after MPP⁺ (MP versus ME: *P*<0.001) (Fig. 1C). In contrast, both MPP⁺ and E-EPA did not affect the DHA content in brain slice tissue (Fig. 1D).

3.4. Omega-6 fatty acid profiles in brain tissue

Two-way ANOVA showed that linoleic acid (C18:2, n–6) was significantly affected by both MPP⁺ ($F[1,31]=5.308$, *P*<0.01) and DIET ($F[1,31]=7.623$, *P*<0.05). The post-hoc tests showed that the linoleic acid content was significantly increased by MPP⁺ compared to controls, but only in the palm oil group (P versus MP: *P*<0.05) (Fig. 2A). Although E-EPA by itself did not affect linoleic acid content, it attenuated the MPP⁺ induced increase in linoleic acid concentration (MP versus ME: *P*<0.05) (Fig. 2A). Similar to linoleic acid, arachidonic acid (C20:4, n–6) was affected by MPP⁺, but not by DIET, although the effect of MPP⁺ only approached significance ($F[1,32]=4.109$, *P*=0.06). Nonetheless, there was a significant interaction between MPP⁺ and DIET ($F[1,32]=6.81$, *P*<0.05). The interaction effect justified a post-hoc test, which demonstrated that MPP⁺ significantly increased arachidonic acid content compared to controls in the palm oil group (P versus MP: *P*<0.01), but E-EPA attenuated this effect (MP versus ME: *P*<0.05), while having no effect by itself (Fig. 2B).

Fig. 1. Brain tissue n-3 fatty acid content. Omega-3 PUFAs were measured with GC from striatum and frontal cortex tissue. A: alpha(α)-linolenic acid (C18:3, n-3): was not affected by any treatment. B: eicosapentaenoic acid (EPA, C20:5, n-3) was strongly increased by E-EPA compared to palm oil, in both the control (P versus E, *** $P < 0.001$) and MPP⁺ condition (MP versus ME, ### $P < 0.001$). C: docosapentaenoic acid (DPA, C22:5, n-3) was also significantly increased by E-EPA in control (P versus E, *** $P < 0.001$) and MPP⁺ conditions (MP versus ME, ### $P < 0.001$). D: docosahexaenoic acid (DHA, C22:6, n-3) was not affected by any treatment. Results are presented as mean $\pm$ S.E.M.

3.5. Striatal mRNA expression of cPLA2 and COX-2

Two-way ANOVA indicated that there was a significant effect of MPP⁺ on cPLA2 mRNA expression ($F_{1,21} = 19.2$, $P < 0.01$, but DIET did not affect the expression of this gene. MPP⁺ increased cPLA2 mRNA expression compared to controls in both the palm oil (P versus MP: $P < 0.05$) and E-EPA (E versus ME: $P < 0.05$) groups (Fig. 3A). The mRNA expression of COX-2 was also affected by MPP⁺ ($F_{1,20} = 4.6$,

$P < 0.05$), but not by DIET. The post-hoc tests revealed that the effect of MPP⁺ on COX-2 was not strong enough to be significant after the Bonferroni correction (Fig. 3B).

3.6. Striatal mRNA expression of bax, bcl-2 and caspase-3

There was no significant main effect of MPP⁺ on bax mRNA expression, but an effect of DIET was detected ($F_{1,121} = 4.649$,

Fig. 2. Brain tissue n-6 fatty acid content. Omega-6 PUFAs were measured with GC from striatum and frontal cortex tissue. A: linoleic acid (C18:2, n-6) was significantly increased by MPP⁺, but only in the palm oil group (P versus MP: * $P < 0.05$). E-EPA attenuated this effect (MP versus ME: # $P < 0.05$). B: arachidonic acid (C20:4, n-6) was also increased by MPP⁺ in the palm oil group (P versus MP: ** $P < 0.01$), but E-EPA attenuated this effect as well (MP versus ME: # $P < 0.05$). Results are presented as mean $\pm$ S.E.M.

Fig. 3. Striatal mRNA expression of cPLA2 and COX-2. Indices of fatty acid metabolism and inflammation were measured by real-time PCR analyses of cPLA2 and COX-2. A: cytosolic PLA2 (cPLA2) was significantly increased by MPP⁺ in both the palm oil (P versus MP: * $P < 0.05$) and E-EPA groups (E versus ME: * $P < 0.05$). B: cyclooxygenase 2 (COX-2) was not significantly affected by treatment. Results are presented as mean $\pm$ S.E.M.

$P < 0.05$). Indeed, E-EPA significantly downregulated bax, but only in the MPP⁺ condition (MP versus ME: $P < 0.05$), while E-EPA by itself (no MPP⁺ treatment) did not have an effect (Fig. 4A). No significant effect of either MPP⁺ or E-EPA was found on bcl-2 (Fig. 4B). MPP⁺ (but not DIET) affected caspase-3 gene expression, although this effect only approached significance ($P = 0.06$). Nonetheless, there was a significant interaction between MPP⁺ and DIET ($F[1,20] = 4.35$, $P = 0.05$). Although MPP⁺ did not significantly increase caspase-3 gene expression compared to controls by Bonferroni analyses, the increase was significant (in the palm oil group) with independent sample *t*-test (P-MP: $P < 0.05$). Bonferroni tests showed that E-EPA attenuated this MPP⁺ induced increase of caspase-3 mRNA expression (MP versus ME: $P < 0.05$) (Fig. 4C).

4. Discussion

The present study investigated whether acute administration of parkinsonian neurotoxin MPP⁺ can cause changes in brain lipids and lipid metabolism that otherwise appear stable in advanced stage Parkinson's disease. Furthermore, it was investigated whether long chain n-3 fatty acid EPA can attenuate acute MPP⁺ neurotoxicity and whether this effect is mediated by altered brain EPA rather than DHA levels. Summarized, our major findings were that acute MPP⁺ increased major n-6 fatty acid arachidonic acid and cPLA2 mRNA expression,

while leaving n-3 fatty acids and COX-2 mRNA expression unaltered. The 6-week pre-treatment with E-EPA significantly increased brain EPA and DPA concentrations rather than DHA. E-EPA pre-treatment resulted in prevention of MPP⁺ induced increases in n-6 fatty acid arachidonic acid, which may be an important mechanism by which EPA prevents initial pro-inflammatory processes induced by MPP⁺.

Repeated systemic administration of MPTP to mice renders a model that mimics an advanced stage of Parkinson's disease, including significant loss of nigrostriatal dopaminergic terminals and cell bodies, and depletion of nigrostriatal dopamine (Dauer and Przedborski, 2003). Acute MPP⁺ administration to striatal brain slices also causes a striatal dopamine depletion, but by excessive release of dopamine from nigrostriatal terminals without sufficient repletion (Wilson et al., 1990; Snape et al., 1988). Presently, the depletion of striatal dopamine after acute MPP⁺ treatment likely occurred by such a release mechanism, since HVA levels and the HVA/dopamine ratios were increased, which may indicate increased extra-cellular dopamine levels (Rabey and Burns, 2002). As striatal tissue was disconnected from the SN in brain slices, MPP⁺ either directly targeted the nigrostriatal terminals (Dauer and Przedborski, 2003), or rather targeted an intrinsic population of dopaminergic inter-neurons within the striatum (Huot et al., 2007). In Parkinson's disease, alterations in non-dopaminergic and extra-striatal neurochemistry are a major aspect of neuropathology (Luchtman et al., 2009; Rommelfanger and Weinschenker, 2007; Rousselet et al., 2003). Acute MPP⁺ administration to frontal cortex slices depleted noradrenaline, suggesting that MPP⁺ also directly affected extra-striatal neurochemistry in the acute stage of neurotoxicity, without prior degeneration in the afferent locus coeruleus, the major noradrenergic nucleus. MPP⁺ enters neurons by means of the monoamine transporter (Pifl et al., 1996). Since the noradrenaline transporter (NET) is denser in the frontal cortex than the dopamine transporter (DAT) (Pifl et al., 1996), this may explain why MPP⁺ affected noradrenaline rather than dopamine in the frontal cortex. These acute effects of MPP⁺ may be similar to the effect of acute exposure to environmental neurotoxins, such as pesticides, that are thought to trigger PD (Di Monte, 2003).

Consistent with our hypothesis, MPP⁺ presently increased n-6 PUFAs linoleic acid and arachidonic acid, and cPLA2 mRNA expression. Counter to our hypothesis through, n-3 fatty acids were not decreased and COX-2 mRNA expression remained unaltered. Since previous studies demonstrated no changes in fatty acid content in non-medicated Parkinson's disease patients, or MPTP treated monkeys (Julien et al., 2006) and mice (*in-vivo*) (Bousquet et al., 2008), the present effect of MPP⁺ on n-6 fatty acids may be transient, but nonetheless an important initial step in Parkinson's disease pathogenesis, since the increase in arachidonic acid contents, combined with increased expression of cPLA2, could increase arachidonic acid substrate for COX-2 metabolism, and trigger inflammation. The cause of the acute MPP⁺ induced increase in n-6 fatty acids remains speculative. Cellular stress, induced by acute administration of MPP⁺, could activate cellular pathways involved in fatty acid synthesis and turnover (Colgan et al., 2007). Furthermore, it is possible that the temporary increase of extra-cellular dopamine, caused by acute MPP⁺ activates D2 receptors, which can activate arachidonic acid synthesis (Huo and Healy, 1991). For instance, when Parkinson's disease patients and MPTP treated monkeys were medicated with L-DOPA, which also increases extra-cellular dopamine, cortical arachidonic acid levels increased as well (Julien et al., 2006). Based on these findings, we propose a hypothesis that an initial increase in arachidonic acid and cPLA2 in neurons following acute MPP⁺ exposure may promote an environment favorable to increased pro-inflammatory eicosanoid production, which may in turn trigger microglia, the major inflammatory cells of the brain (Jin et al., 2007). It is possible that apoptosis genes were not significantly activated due to insufficient neurotoxicity caused by the single acute dose of MPP⁺ and/or too early measurement of gene expression following

Fig. 4. Striatal mRNA expression of bax, bcl-2 and caspase-3. Indices of apoptosis were measured by real-time PCR analyses of bax, bcl-2 and caspase-3. A: bax was not affected by MPP⁺. Ethyl-EPA downregulated bax mRNA expression compared to palm oil, but only in the MPP⁺ condition (MP versus ME: $P < 0.05$). B: bcl-2 was not affected by either MPP⁺ or diet. C: caspase-3 was marginally increased by MPP⁺ and this effect was attenuated by E-EPA treatment (MP versus ME: $P < 0.05$). Results are presented as mean $\pm$ S.E.M.

administration of the neurotoxin. It should be noted that dendritic neurodegeneration has been observed in brain slices after 2 h of MPP⁺ 50 μ M, but it is unclear whether these effects were due to apoptosis (Bywood and Johnson, 2003).

Increasing evidence has suggested that n–3 fatty acids may provide major benefits compared to the traditional pharmacotherapy for Parkinson's disease, such as L-DOPA. Most studies, however, focused on the effects of the major brain n–3 fatty acid DHA (Delattre et al., 2010; Bousquet et al., 2008, 2009). Even though the content of EPA in the brain is in the undetectable range (Kawashima et al., 2009; Vancassel et al., 2007) or in extremely low quantities (Bousquet et al., 2008 and presently observed), it is a neuroactive lipid that, when supplemented, has proven benefits in affective disorders and schizophrenia (Horrobin et al., 2002). As such, EPA may also help to treat non-motor symptoms of Parkinson's disease, such as depression and anxiety (Peet and Stokes, 2005; Horrobin et al., 2002). In the present study, it was observed that EPA exerted modest beneficial effects against acute MPP⁺ induced neurotoxicity. The exact mechanisms by which EPA exerts beneficial effects are unknown, but our present findings suggested that the effects of E-EPA were not mediated by DHA, but rather by EPA or its metabolite DPA (C22:5, n–3). E-EPA increased tissue EPA content to a similar degree as previously reported following highly purified E-EPA (Willis et al., 2009) or EPA/DHA administration (Bousquet et al., 2008). Similar to Willis et al. (2009), tissue DPA content (C22:5, n–3) was also increased, while DHA was not affected. These findings may suggest that DPA could play an important role in the effects of E-EPA feeding observed in this study. Since the functional significance of DPA in the brain has not yet been determined, it warrants further investigation. E-EPA supplementation did not affect n–6 fatty acid content. Generally, a decrease of n–6 fatty acids is observed after dietary supplementation with n–3 fatty acids, due to displacement (Horrobin et al., 2002; Chalon et al., 1998), but only when very high doses of n–3 fatty acids are used (above 1%) (Horrobin et al., 2002). Nonetheless, and more importantly, when brain slices were treated with MPP⁺, E-EPA fully attenuated the increase of linoleic acid and arachidonic and thus reduced the availability of arachidonic acid substrate for COX-2 metabolism. Although the diet did not attenuate the increase in cPLA2 mRNA expression, these results suggest that the diet promoted an environment less favorable to inflammation. It should be noted that in previous studies, a similar EPA diet suppressed cPLA2 expression or activity (Song et al., 2007), but the toxin used (Interleukin-1 β) was probably milder than the present MPP⁺.

Another important mechanism by which n–3 fatty acids could protect in Parkinson's disease is by modulating neurochemistry. Consistent with previous observations that diets either deficient or enriched with n–3 fatty acids can alter neurochemistry (Chalon, 2006), E-EPA by itself (no MPP⁺ treatment) presently increased dopamine content in the frontal cortex, while noradrenaline, or dopamine turnover was not affected. The precise mechanisms by which n–3 fatty acids affect neurochemistry are not fully known, but likely involve structural alterations at the level of the neuronal membrane, neurotransmitter storage, release and metabolism (Chalon, 2006). E-EPA modestly ameliorated MPP⁺ dopaminergic toxicity by reducing striatal dopamine turnover and partially preventing a reduction in frontal cortex noradrenaline. Delattre et al. (2010) also found that n–3 PUFA supplementation merely affected dopamine turnover following parkinsonian neurotoxin exposure.

5. Conclusions

The present study demonstrated that 1) brain n–6 fatty acids are altered in the acute stage of exposure to parkinsonian neurotoxin and 2) that n–3 fatty acid EPA rather than major n–3 fatty acid DHA can modulate changes in neurochemistry and brain lipids induced by the neurotoxin. Furthermore, we demonstrated that MPP⁺ can directly

affect frontal cortex neurochemistry in the acute stage. Our findings suggest that changes in brain lipids in the acute stage of exposure to parkinsonian neurotoxins may promote pro-inflammatory conditions. Furthermore, our findings are consistent with previous studies demonstrating that EPA, despite its low membrane content, may provide beneficial effects in Parkinson's disease. However, since EPA did not counteract the depletion of dopamine caused by MPP⁺, perhaps longer diet duration would increase the benefit obtained from this n–3 fatty acid.

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