Isotopic reinforcement of essential polyunsaturated fatty acids diminishes nigrostriatal degeneration in a mouse model of Parkinson's disease

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A B S T R A C T

Oxidative damage of membrane polyunsaturated fatty acids (PUFA) is thought to play a major role in mitochondrial dysfunction related to Parkinson’s disease (PD). The toxic products formed by PUFA oxidation inflict further damage on cellular components and contribute to neuronal degeneration. Here, we tested the hypothesis that isotopic reinforcement, by deuteriation of the bisallic sites most susceptible to oxidation in PUFA may provide at least partial protection against nigrostriatal injury in a mouse model of oxidative stress and cell death, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model. Mice were fed a fat-free diet supplemented with saturated acids, oleic acid and essential PUFA: either normal, hydrogenated linoleic (LA, 18:2n−6) and α-linolenic (ALA, 18:3n−3) or deuterated 11,11-D2-LA and 11,11,14,14-D4-ALA in a ratio of 1:1 (to a total of 10% mass fat) for 6 days; each group was divided into two cohorts receiving either MPTP or saline and then continued on respective diets for 6 days. Brain homogenates from mice receiving deuterated PUFA (D-PUFA) vs. hydrogenated PUFA (H-PUFA) demonstrated a significant incorporation of deuterium as measured by isotope ratio mass-spectrometry. Following MPTP exposure, mice fed H-PUFA revealed 78.7% striatal dopamine (DA) depletion compared to a 46.8% reduction in the D-PUFA cohort (as compared to their respective saline-treated controls), indicating a significant improvement in DA concentration with D-PUFA. Similarly, higher levels of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were detected in MPTP-exposure mice administered D-PUFA; however, saline-treated mice revealed no change in DA or DOPAC levels. Western blot analyses of tyrosine hydroxylase (TH) confirmed neuroprotection with D-PUFA, as striatal homogenates showed higher levels of TH immunoreactivity in D-PUFA (58.5% control) vs. H-PUFA (50.4% control) in the MPTP-treated cohorts. In the substantia nigra, a significant improvement was noted in the number of nigral dopaminergic neurons following MPTP exposure in the D-PUFA (79.5% control) vs. H-PUFA (58.8% control) mice using unbiased stereological cell counting. Taken together, these findings indicate that dietary isotopic reinforcement with D-PUFA partially protects against nigrostriatal damage from oxidative injury elicited by MPTP in mice.

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Abbreviations: PD, Parkinson’s disease; LA, linoleic acid; ALA, linolenic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PUFA, polyunsaturated fatty acid; D-PUFA, deuterated-PUFA; H-PUFA, hydrogenated PUFA; TH, tyrosine hydroxylase; HNE, 4-hydroxynonenal; HHE, 4-hydroxhexenal; MAO, monoamine oxidase; ROS, reactive oxygen species; RCP, reactive carbonyl product.

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1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease, affecting 1–2% of the population over 65 years of age (Lees et al., 2009). A role for oxidative stress in PD etiology is supported by a vast literature, including evidence of polyunsaturated fatty acid (PUFA) peroxidation products, including 4-hydroxynonenal (HNE) and malondialdehyde (MDA), the major products of PUFA oxidation in post-mortem tissue for patients with PD (Cookson and Bandmann, 2010; Zhu and Chu, 2010; Hirsch, 1993; Ischiropoulos and Beckman, 2003; Jenner, 2003; Loh et al., 2006; Fukae et al., 2007; Henchcliffe and Beal, 2008; Miyazaki and Asanuma, 2008; Navarro and Boveris, 2009). Furthermore, oxidized PUFA initiate further cellular injury through oxidation of DNA, proteins and other cellular targets, including those linked to PD: dopamine (DA) and importantly alpha-synuclein (Chau et al., 2010; Qin et al., 2007; Parahar et al., 2008; Hattoria et al., 2009; Lee et al., 2009; Ubbi et al., 2009).

PUFA are essential components that make up a substantial fraction of lipid membranes. PUFA, specifically linoleic (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) acids, are early targets of oxidation by reactive oxygen species (ROS) (Fig. 1). This damage deteriorates the properties of lipid membranes such as fluidity (Dobretsov et al., 1977). Moreover, oxidized PUFA can damage other biomolecules, most notably proteins and DNA, through reactive carbonyl products (RCP) such as HNE, 4-hydroxyhexenal (HHE), malondialdehyde (MDA) and acrolein (Lim et al., 2004).

Mitochondrial membranes contain a high content of PUFA, compared to other cellular membranes. In mitochondria, the phospholipid cardiolipin accounts for 18% of the total phospholipids and is rich in linoleic acid; indeed, within cardiolipin, 90% of lipid moieties are unsaturated (Daum, 1985; Bindoli, 1988). Lipid oxidation in mitochondria, especially peroxidation of cardiolipin, is associated with apoptosis (Orrenius, 2007) and aging (Bindoli, 1988). In fact, cardiolipin oxidation may be a critical factor that initiates apoptosis by reducing the binding of cytochrome c to the mitochondrial inner membrane and facilitating permeabilization of the outer membrane (Orrenius, 2007; Schug and Gottlieb, 2009). Cytochrome c, in turn, activates a proteolytic cascade that culminates in apoptotic cell death (Danial and Korsmeyer, 2004; Schug and Gottlieb, 2009). Previous studies have examined the efficacy of compounds aimed at reducing or neutralizing ROS; however, the antioxidant strategies have had limited success (Halliwell, 2011). The impact of stabilization of biological molecules subject to ROS attack (i.e. PUFA) has not been investigated. In this study, we evaluated whether neuroprotection is elicited in a model of oxidative stress and neurodegeneration, through PUFA stabilization by substituting deuterium for hydrogen at oxidation-prone bisallylic sites of these essential fatty acids. We found that dietary supplementation with deuterated PUFA (D-PUFA) led to brain incorporation of deuterium and partial protection of the nigrostriatal dopaminergic pathway following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) challenge to mice.

2. Materials and methods

2.1. D-PUFA synthesis

D-PUFA (Fig. 1B), obtained by several step syntheses on a 30–40 g scale as described in detail previously (Tucker and White, 1970; Meyer and Kleinman, 2008; Hill et al., 2011), were fully characterized by 1H and 13C NMR, MS and GC. The compounds were approximately 97% isotopically pure and used as free acids. Non-deuterated PUFA were obtained from Sigma–Aldrich (99%; St. Louis, MO, USA). Both D- and H-PUFA were stored under nitrogen at 0°C to reduce autoxidation.

2.2. Animals, diet and D-PUFA administration

Mice (C57BL/6; aged 8 weeks; Charles River Labs; Gilroy, CA, USA) were maintained on a 12 h light–dark cycle and given food and drinking water ad libitum. All animal procedures and care methods were approved by the Institutional Animal Care and Usage Committee for the National Institutes of Health, SRI International and the Parkinson’s Institute. Mice were individually housed in a diet pellet (5 g/mouse/day) of MPBiO Fat Free Mouse Diet AIN 76A (Cat. # 960321). To 95 g of this diet, the following fat components (9 g in total) were added: (i) saturated fatty acids (6.6 g total) containing a mixture of palmitate: 4.8 g (Sigma–Aldrich 95%); stearate: 1.2 g (Sigma–Aldrich 95%); myristate: 0.6 g (Sigma–Aldrich 98%) and (ii) the monounsaturated oleic acid (2.4 g total; Sigma–Aldrich 93%). The PUFA component of the diet (0.8 g (0.8%) LA and 0.8 g (0.8%) ALA per 100 g diet in a 1:1 ratio) was delivered by daily application to food pellet; pellets were supplemented with either control PUFA (i.e. hydrogen–H-PUFA) or experimental PUFA (i.e. deuterium, D-PUFA). The total combined fat composition of the diet was 9.7% (saturates, monounsaturates and PUFA). Mice were fed either D- or H-PUFA-supplemented diet for six days prior to toxicant or vehicle administration and in the days following administration until sacrifice.

2.3. MPTP administration

After mice were fed a diet supplemented with H-PUFA (n = 8) or D-PUFA (n = 8) for six day, either MPTP or saline was administered to a subset from each cohort. Mice (n = 4 from each group) received a single i.p. injection of 40 mg/kg MPTP (Sigma–Aldrich) in saline or the vehicle alone, and killed six days later. In another experiment, mice were supplemented with D- or H-PUFA as described above (n = 6 per group) were injected with 20 mg/kg MPTP or saline (n = 2–3 per group) for a single injection and killed one week later.

2.4. Tissue processing

Trunk blood was collected and sent to the University of California–Davis Comparative Pathology Laboratory to measure biomarkers of pancreas, kidneys and liver function in H- and D-PUFA mice. Brains were removed and blocked to separate forebrain from mid- and hind-brain. For dopamine and metabolite determination, striatal (from both hemispheres) were dissected on ice and placed in 0.4 M perchloric acid (Sigma–Aldrich). For deuterium incorporation and Western blot analyses, dissected brain regions were frozen on dry ice until needed (Manning-Bog et al., 2002; Pursnai et al., 2005). For immunohistochemistry, the tissue block containing mid- and hind-brain from each mouse was immersion fixed in 4% paraformaldehyde and successively cryoprotected in 10 and 30% sucrose over the course of 72 h (Manning-Bog et al., 2002, 2003). Brains were cryostat-cut into 40 μm sections, and the entire extent of the substantia nigra was collected into microcentrifuge tubes at 240-μm intervals and stored in cryopreservative solution at −20°C until needed.

2.5. Deuterium incorporation

From all four cohorts, D-PUFA–saline, D-PUFA–MPTP, H-PUFA–saline and H-PUFA–MPTP (n = 4 per group), brain regions were dissected, processed and maintained at −80°C until use as described above. Forebrain was thawed on ice and sonicated briefly; aliquots of tissue samples from each brain (10 mg wet weight) were lyophilized for deuterium evaluation using isotope ratio mass spectrometry (iMS) (Caire et al., 2002). Samples (100 μg) were placed into silver capsules (Costech; Valencia, CA, USA) to avoid contamination by hydrogen. Capsules were loaded into a Costech Zero Blank Auto-sampler connected to a Thermo/Finnigan thermochromical elemental analyzer (TCEA)/Thermo Fisher (Bremen, Germany), and pyrolyzed at 1400°C to H2 gas which was injected into a Thermo Electron Corp. Delta V Plus iMS via a ConFlo III combustion interface (Thermo Fisher). After each sample was analyzed in triplicate, δD values were normalized to the Vienna standard mean ocean water (VSMOW) scale (approx 150 ppm) using different concentrations of D2-LA and D4-ALA controls in a matrix of processed forebrain from untreated mice. Data are shown as δD (in permille (‰)), where $\delta^{D} = (R_{sample}/R_{standant}) \times 1000$; with K being the ratio of the abundances of deuterium and protium, K = D/H.

2.6. Neurochemistry

Catecholamines and their metabolites were quantified by reverse phase HPLC coupled with electrochemical detection (EC). Briefly, catecholamines were extracted in 0.4N perchlorate from striatal homogenates by sonication and centrifuged as previously described (Manning-Bog et al., 2007). The supernatant was removed and stored at −80°C until use for DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) determinations. The supernatant was replaced by an aqueous solution of 0.5M NaOH for protein determination. Samples and external standards were eluted at a flow rate of 1 ml/min from a C18 column (Luna C18(2); Phenomenex; Torrance, CA, USA) and matching precolumn. The elution order and retention times of the catecholamines were as follows: DOPAC (1.1 min), DA (1.44 min) and HVA (25.7 min) (Klippe et al., 1986; Manning-Bog et al., 2007). The compounds were measured using EC detector (ESA Inc.; Chelmsford, MA, USA). The chromatograms were recorded and integrated using manufacturer-supplied proprietary software (32 Karat Software; Beckman Coulter, Inc.; Fullerton, CA, USA).
2.7. Western blot analyses

Striatal tissues were homogenized in 10 mM Tris/1 mM EDTA/protease and phosphatase inhibitor cocktails (1:1000; Sigma-Aldrich) by sonication and subsequently centrifuged at 10,000 × g for 10 min. The supernatant was decanted. The protein concentration was measured using BCA protein concentration determination kit (Thermo Fisher, Rockville, IL, USA). Protein samples were applied to nitrocellulose using a vacuum blotter; the blots were blocked and incubated overnight at 4 °C with anti-tyrosine hydroxylase (Pel Freez Biologicals, Rogers, AR) or anti-tubulin (Sigma-Aldrich). Appropriate secondary antibodies conjugated to HRP were applied, and blots were incubated with a chemiluminescent substrate (Thermo Fisher) and exposed to Kodak X-Omat Blue Film (Kodak; Rochester, NY, USA) (Manning-Bog et al., 2002, 2003, 2007).

2.8. Stereology and immunohistochemistry

Sections from midbrain sampling the entire extent of the substantia nigra were immunostained using an antibody against TH as previously described (Manning-Bog et al., 2003, 2007). Sections processed for TH immunohistochemistry were counterstained with 0.5% Cresyl violet to demonstrate the nuclei. Sections for HPS60 histochemistry were blocked in 3% normal donkey serum, 1% BSA and 0.1% TX-100 in PBS and incubated with an antibody generated in goat against HPS60 (Santa Cruz Biologicals; Santa Cruz, CA, USA) followed by donkey anti-goat secondary antibody conjugated to FITC (Jackson Immunoresearch Laboratories, Inc.; West Grove, PA, USA). On TH-labeled sections, stereological analysis was performed using an Olympus BX2 microscope with a motorized X-Y stage linked to a computer-assisted stereological system (Castrigard; Olympus; Albertslund, Denmark). The sections were delineated as previously described (McCormack et al., 2002) and the substantia nigra systematically sampled at 100× magnification. Sections were examined in the z direction through the optical plane/disector at 8 μm with a guard range of 2 μm. The total number of dopaminergic neurons was estimated using the optical fractionator technique for an unbiased estimate of total number independent of size, shape and shrinkage (McCormack et al., 2002; Manning-Bog et al., 2003, 2007).

2.9. Statistical analyses

Differences among means were analyzed using one-way analysis of variance (ANOVA) and post-hoc analysis was employed using Tukey-Kramer testing when differences were observed in ANOVA testing (p < 0.05).

3. Results

3.1. Dietary effects

Mice were treated with linoleic (18:2, n – 6 [LA]), and α-linolenic (18:3, n – 3 [ALA]) acids hydrogenated (H-PUFA) or deuterated (D-PUFA) at bis-allylic positions (Fig. 1B). Published protocols were used to synthesize 11,11-D2-LA and 11,11,14,14-D4-ALA (Hill et al., 2011; Tucker and White, 1970; Meyer and Klinman, 2008). The structures and purity of the deuterated PUFA were confirmed by 1H and 13C NMR, MS and GC (Hill et al., 2011).

Mice readily consumed a fat-free diet supplemented with either D-PUFA or H-PUFA. Both groups gained weight normally with no significant difference between the cohorts (data not shown).

3.2. Brain deuterium incorporation

Lyophilized forebrain homogenate samples from saline- and MPTP-treated mice were evaluated for deuterium content using ir-MS and compared with the levels in the deuterium standard, V-SMOW and measured in ‰ per mil (Table 1). Lipids, due to reduced hydrogen exchange and some aspects of their biosynthesis (Valentine, 2009), are depleted in deuterium relative to other biomolecules and the V-SMOW standard. Indeed, the negative values from samples from H-PUFA-fed mice noted in Table 1 reflect this fact.

Using the permil ‰, or 0.1%, scale (i.e. the scale most commonly utilized for isotope composition measurements), mice fed the D-PUFA-supplemented diet demonstrated an approximate 14,000 (‰) increase over H-PUFA-fed mice. Thus, a 14-fold increase in brain deuterium incorporation was revealed in D- versus H-PUFA-treated cohort (Table 1). Further, consistent deuterium incorporation was detected in brain from both the MPTP- and saline-treated mice, with no significant difference in incorporation detected between the two groups and thus allowing comparisons to be made between these cohorts.

3.3. Blood biomarkers of toxicity

Serum obtained from H- and D-PUFA-supplemented mice was evaluated for blood indicators of renal and liver functions. No significant difference in samples from for H- vs. D-PUFA-fed animals was observed in albumin (3.04 ± 0.33 vs. 3.17 ± 0.3 g/dl), total protein (6.29 ± 0.29 vs. 5.83 ± 0.22 g/dl), total bilirubin (0.288 ± 0.05 vs. 0.220 ± 0.05 mg/dl) or blood urea nitrogen (23.7 ± 8.0 vs. 22.8 ± 4.6 mg/dl). No significant difference was noted in blood

| Table 1 | Brain deuterium incorporation following dietary D- or H-PUFA supplementation. |
|---------|-----------------------------|-------------------|------------------|----------------|-----------------|
| Group   | D/H (‰ permil)             |                   |                  |                |                 |
| H-PUFA-saline | −124.75 ± 17.90            |                  |                  |                |                 |
| H-PUFA-MPTP | −120.62 ± 0.51             |                  |                  |                |                 |
| D-PUFA-saline | 14455.83 ± 912.79          |                  |                  |                |                 |
| D-PUFA-MPTP  | 13413.00 ± 1245.71         |                  |                  |                |                 |
glucose or lipid profiles (e.g., triglycerides and non-esterified fatty acid) as well (data not shown).

3.4. Sensitivity to striatal injury by MPTP

To determine whether deuterium-for-hydrogen isotopic replacement within PUFA may, at least in part, protect against oxidative injury and neurodegeneration, MPTP nigrostriatal toxicity was compared in mice fed H- vs. D-PUFA. Mice were acutely injected with either saline or a robust (40 mg/kg) MPTP dose. Mice continued on the H- or D-PUFA diet until sacrifice six days later. To establish that isotopic reinforcement does not interfere with DA synthesis, its metabolism or the metabolism of MPTP, we examined DA, DOPAC and HVA values in mice fed H- or D-PUFA and administered saline. In saline-treated mice, no significant difference was detected in DA or its metabolites DOPAC or HVA between the H- and D-PUFA-fed groups (Fig. 2A).

In H-PUFA-supplemented mice administered 40 mg/kg MPTP, toxicant exposure caused a loss of 78.8% DA, 64.3% DOPAC and 30.9% HVA as compared to H-PUFA-fed, saline-treated mice. The D-PUFA-fed cohort was significantly less affected by MPTP which caused a 46.8%, 36.5% and 26.9% depletion of DA, DOPAC and HVA, respectively, as compared to the D-PUFA cohort injected with saline (Fig. 2A).

Similar findings of neuroprotection were observed in a preliminary experiment using a modest MPTP lesion. Following 6 days of H- or D-PUFA supplementation, mice were administered either saline or 20 mg/kg MPTP and sacrificed a week later. No difference in striatal DA or metabolite concentrations were detected between D- or H-PUFA-fed mice injected with saline. While H-PUFA fed, MPTP-treated mice had depletion of striatal DA and DOPAC (14.0% and 13.6%, respectively), D-PUFA-administered, MPTP-exposed mice were completely protected from the lesion (data not shown).

To validate the neurochemical findings, we performed Western blot analysis in striatal homogenate for immunoreactivity for the rate-limiting enzyme for DA synthesis, TH. Indeed, following MPTP exposure, D-PUFA-supplemented animals revealed significantly higher levels of striatal TH immunoreactivity (63.6% of the D-PUFA saline group) compared to those fed H-PUFA (36.5% of the H-PUFA saline group). Immunoreactivity for TH was not significantly different between the saline-treated cohorts (Fig. 2B).

3.5. Sensitivity to nigral injury by MPTP

Unbiased stereological cell counting of nigral total (i.e. Nissl stained) and dopaminergic (i.e. TH-positive) neurons was performed to determine whether neuroprotection extended to the level of the substantia nigra. No change was detected in H- vs. D-PUFA-supplemented mice injected with saline. Significantly higher numbers of nigral dopaminergic neurons were revealed in the D-PUFA (79.5% control) vs. H-PUFA (58.8% control) mice following MPTP administration (Fig. 2C). This increase in TH-positive neurons in the D-PUFA- vs. H-PUFA-treated MPTP-challenged mice, as shown in Fig. 2D, indicates a rescue of the dopaminergic phenotype using isotopic reinforcement with D-PUFA. Interestingly, evaluation of the mitochondrial marker Hsp60 revealed that, following MPTP exposure, a robust increase in protein level was apparent within the substantia nigra in sections from D-vs. H-PUFA treated mice (Fig. 3).

4. Discussion

Although previous studies have indicated that non-deuterated PUFA supplementation elicits protects against CNS degeneration (Bousquet et al., 2010; Rodriguez de Turco et al., 2002; King et al., 2006), our approach here addresses a unique question: whether isotopic reinforcement of PUFA prevents oxidative stress-related injury in the nigrostriatal pathway. We took advantage of the essential nature of PUFA (i.e. must be obtained from diet) which ensures delivery of this material throughout the body, including across the blood brain barrier, and provided mice with either D-PUFA or H-PUFA through dietary supplementation. Analyses of brain deuterium by ir-MS confirmed significant incorporation of the isotope in the D-PUFA-fed mice that did not differ significantly between saline- and MPTP-challenged mice (Table 1). Neurochemical, biochemical and unbiased stereological analyses all supported the idea that isotopic reinforcement may represent a potential protective therapy as striking neuroprotection of striatal DA values, striatal TH levels and nigral dopaminergic neurons was observed (Fig. 2). These studies revealed no obvious effect on growth and weight maintenance, good blood–brain barrier transit, and most importantly, for the first time demonstrate pre-efficacy of isotopically reinforced PUFA in a mouse model of oxidative stress-induced injury.

PUFA are delivered to the brain to replenish normal tissue homeostasis. Importantly, our studies revealed oral delivery of deuterium-reinforced PUFA is sufficient to elicit a CNS-mediated response. Furthermore, the finding of enhanced levels of deuterium indicates D-PUFA incorporation in the brain. Thus, given the normal turnover of PUFA, and literature references of 3–8% incorporation in brain per day (Rapoport et al., 2001), we anticipate that, in our model, D-PUFA accounts for approximately 30% of total brain PUFA following 12 days of D-PUFA supplementation. In the robust MPTP paradigm, this level of incorporation resulted in a near 3-fold improvement in striatal DA levels as determined by neurochemical measurements.

It is critical to point out that there were no significant changes in the major biomarkers of renal function, liver function, blood glucose and blood lipids between the H- and D-PUFA-treated cohorts from our studies. These data suggest that D-PUFA administration in this paradigm does not produce adverse toxic changes in systemic markers. Our findings are consistent with previous studies of rodent models dosed with D-PUFA that demonstrated no abnormalities in tissues analyzed (Lin and Salem, 2007). Deuterated fatty acids including per-deuterated compounds have been utilized in numerous human studies as well, with doses as high as multiple grams per day, for decades in some instances, with no reported ill effects on biochemistry, histopathology or toxicology (Emken et al., 1989, 1990, 1992, 1994, 1998; Pawlosky et al., 2001, 2003, 2009; Votrubová et al., 2001). Indeed, deuterated PUFA have been administered even to lactating women and growth-impaired infants (Caire et al., 2002; Lin et al., 2010; Emken et al., 1989; Salem et al., 1996; Pawlosky et al., 2006). Taken together, the lack of adverse effects in these studies indicate that supplementation with D-PUFA may represent a safe therapeutic option.

Drugs aimed at reducing or neutralizing ROS have been extensively evaluated with little success in disease modification for PD (Halliwell, 2011), but no therapeutic approach has employed stabilization of the biological molecules first subject to ROS attack, PUFA (Fig. 1A). Our findings here are an important initial step in determining the utility of this approach by providing proof-of-concept data that D-PUFA elicits neuroprotection in a mouse model that mimics the oxidative stress and nigrostriatal degeneration of PD. Although the mechanism by which D-PUFA plays a protective role is not fully elucidated, our studies suggest that protection at the level of the mitochondria is involved (Fig. 3). Hsp60 has been implicated in cellular apoptosis, playing both anti- and pro-apoptotic roles under certain conditions (Kirchhoff et al., 2002; Chandra et al., 2007; Campanella et al., 2008), but has been shown to be critical for the maintenance of normal mitochondrial function, particularly following oxidative injury (Veereshwarayya et al., 2006). Indeed, previous studies in cardiac myocytes have revealed a correlation between decreased cytochrome c release...
Fig. 2. D-PUFA treatment diminishes nigrostriatal injury. Striatal DA, DOPAC and HVA was significantly reduced following MPTP challenge in both H-PUFA- (open bars) and D-PUFA- (black bars) treated mice (*p < 0.05). D-PUFA treatment significantly reduced the MPTP-induced lesion in striatal DA and DOPAC as compared to H-PUFA treatment (*p < 0.05) (A). Immunoreactivity for TH in striatal homogenates was significantly reduced following MPTP challenge in both H-PUFA- (open bars) and D-PUFA- (black bars) treated mice (*p < 0.05). D-PUFA treatment significantly reduced the MPTP-induced depletion of striatal TH as compared to H-PUFA treatment (*p < 0.05) (B). Stereological cell counts of dopaminergic neurons in substantia nigra revealed a significant depletion in both H-PUFA- (open bars) and D-PUFA- (black bars) treated mice following MPTP challenge (*p < 0.05). D-PUFA administration significantly reduced the loss of TH immunoreactivity in neurons as compared to the H-PUFA treatment group (*p < 0.05) (C). This finding is illustrated in representative images of TH immunostaining in the substantia nigra from mice from each treatment group; bar = 50 µm (D).

and enhanced HSP60 levels (Lin et al., 2007). One interpretation of our data therefore is that mitochondrial integrity and response to MPTP-induced oxidative stress is maintained with D-PUFA treatment, and this may represent a mechanism that contributes to the protective response within the nigrostriatal pathway. Future experiments, however, are clearly warranted to identify the precise mechanisms involved though it is highly plausible that isotopic reinforcement reduces PUFA autoxidation (Shchepinov, 2007). By substituting deuterium-for-hydrogen at oxidation-prone sites, PUFA are 'isotopically reinforced', slowing the rate-limiting step
of PUFA autoxidation, hydrogen abstraction (Elison et al., 1961; Belleau and Moran, 1963). Indeed, such modification has been demonstrated to dramatically reduce autoxidation (i.e. by many fold) and diminish toxic hydroperoxide and RCP formation (Hill et al., 2011; Belleau and Moran, 1963; Shchepinov, 2007). It is important to note that RCP generated by autoxidation of LA and ALA acids include HNE, HHE and MDA that damage DNA, membranes and proteins (including alpha-synuclein), and these events contributes to pathogenesis in PD. Regardless of whether mitochondrial dysfunction/oxidative injury is causal or a contributing factor to PD (Andersen, 2004), the strategy reported here targets a mechanism known to be involved in disease progression. Thus, it is possible that isotopic PUFA fortification represents a potentially safe, easy and effective method that may slow these toxic oxidative events and thus provide modification of disease course.

Conflict of interest statement
M.S.S., C.R.C. and R.J.M. are share-holders of Retrotepe, Inc.

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