Tissue and Cell 56 (2019) 41–51

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

The effect of docosahexaenoic acid on apelin distribution of nervous system in the experimental mouse model of Parkinson's disease

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ARTICLE INFO

Keywords:
DHA
Parkinson
Apelin
Cerebrum
Cerebellum
Pons

ABSTRACT

Parkinson's disease (PD) is a degenerative disorder of the human central and peripheral nervous systems. n-3 fatty acids docosahexaenoic acid (DHA, C22: 6n-3) and eicosapentaenoic acid (EPA) as well as apelin have anti-inflammatory effects in various cells. At the same time, apelin has anti-oxidative and anti-apoptotic effects. The study was conducted to determine the effect of DHA on the distribution of apelin and apelin receptor (APJ) in the central nervous system in 1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine (MPTP)-induced PD model.

DHA treatment decreased the return time and total down time in the Parkinson group which were measured by pole test. Besides, the ambulatory activity, distance and total locomotor activity were increased by DHA in the PD model of animals. The time mice remained on the rotating rod mile was also significantly increased by DHA treatment in MPTP injected animals. The apelin expression in the pons of mice in the Parkinson, DHA and Parkinson + DHA groups were lower compared to the Control group. When apelin and apelin receptor expressions in cerebrum were examined, there was no statistically significant difference between the groups. When apelin receptor expression in cerebellum was examined, the difference between the Control and Parkinson + DHA, Parkinson and Parkinson + DHA, DHA and Parkinson + DHA groups were statistically significant. Apelin receptor expressions in pons of the Parkinson, DHA and Parkinson + DHA groups were lower compared to the Control group. Apelin protein levels of cerebellum and pons were found to be decreased in DHA group compare with Control group.

In conclusion; DHA has been implicated in the expression of the apelin receptor and has reduced the expression of APJ receptor.

1. Introduction

Parkinson's disease (PD) is a progressive and the most common neurodegenerative disorder after Alzheimer's disease (Fahn, 2010). Approximately 1–3% of the population at 65–70 years of age is affected by PD (de Rijk et al., 2000; Nussbaum and Ellis, 2003). Major clinical motor symptoms are rigidity, tremor, bradykinesia and postural instability (Jankovic, 2008; Kidd, 2000; Lees et al., 2009). Nonmotor disorders are depression, sleep loss, fatigue, cognitive disorders and autonomic dysfunction (Aarsland et al., 2017; Gao et al., 2017; Huot, 2015; Ponsen et al., 2009; Yao et al., 2015). PD is induced by progressive loss of dopaminergic neurons in the pars compacta part of the substantia nigra (SNpc) and the depletion of dopamine in corpus striatum. Motor and nonmotor symptoms of PD become apparent after the death of more than 80% of dopaminergic neurons (Groenewegen, 2003; Halliday and McCann, 2010). Until recently, the neurodegeneration of PD is thought to be limited to dopaminergic cell die in substantia nigra (SN) however, neuropathology occurs in the other brain regions, including locus coeruleus, raphe nucleus, dorsal motor nucleus of the vagus, cerebral cortex and also autonomic nervous system (Hou et al., 2017; Yao et al., 2015). Studies have shown that inflammatory changes and oxidative stress are important in inducing PD (Blesa et al., 2015; Chen et al., 2012). The brain is susceptible to oxidative damage because of the excessive polyunsaturated fatty acids (PUFA) content and relatively low antioxidant activity. It has been demonstrated that most of the oxidative destruction in PD is in the SN (Naudi et al., 2017; Smesny et al., 2015). For this reason, studies on the mechanisms of neurodegeneration such as mitochondrial dysfunction and complex I inhibition in PD has focused on this brain region (Hewitt and Whitworth, 2017; Naudi et al., 2017). However, oxidative destruction

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https://doi.org/10.1016/j.tice.2018.12.002
Received 5 September 2018; Received in revised form 19 November 2018; Accepted 5 December 2018
Available online 06 December 2018
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has been also reported in the other brain regions and peripheral tissues of PD patients (Malek et al., 2014).

PUFAs are classified into two categories: n-6 PUFAs and n-3 PUFAs. Linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA 18:3 n-3), respectively, are required for normal cellular functions and are members of the essential fatty acid class (Youdim et al., 2000). The abbreviations "n-6″ and "n-3″ used for unsaturated fatty acids reflect the location of the first double bond after the methyl carbon atom on the distal end of the fatty acid chain (Youdim et al., 2000). Both primary essential fatty acids are required for the synthesis of the long chain PUFA (LCPUFA). In vivo, these PUFAs are metabolised by a series of elongation, desaturation and β-oxidation processes (Laye et al., 2018). These reactions are carried out by elongase and desaturase enzymes in microsome and peroxisomes (Sprecher, 2000). Linoleic acid is the precursor of the arachidonic acid (AA, 20:4 n-6), whereas α-linolenic acid is the precursor of the eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). DHA is a fundamental fatty acid required for neuronal structure and signaling (Bazan et al., 2011). Normally, DHA is located at the phospholipids of neuronal membranes. In cases such as cerebral inflammation and/or ischemia-reperfusion, DHA is separated from membrane phospholipid to the cytoplasm by phospholipase A_2 (PLA2) being subsequently transformed into neuroprotectin D1 (NPD-1) by 15-lipoxygenase enzyme (Bazan, 2006; Echeverria et al., 2017). On the other hand, essential fatty acids regulate the membrane fluidity and lipid-protein interactions and as integral components of the phospholipid layer of the cell membrane. Because of their effects on the cell membrane, they affect the function of transport proteins, hormones and neurotransmitters (Roche, 1999; Yu et al., 2002). It has been identified in studies that the primary LCPUFA of the brain phospholipids is docosahexaenoic acid (Youdim et al., 2000). It has been demonstrated that DHA is effective in inhibiting Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) catalyzed prostaglandin (PG) synthesis which is activated in response to PLA_2 activation. COX-2 activation is also associated with ischemia, oxidative stress, inflammation and the expression of neuroactive cell signaling molecules (Mattammal et al., 1995; McLaughlin et al., 2006). In addition, DHA is important molecule in intracellular signaling pathways (Serhan, 2014). It inhibits tau hyperphosphorylation and regulates the PI3K/Akt pathway (Akbar and Kim, 2002; Green et al., 2007). In addition to its anti-inflammatory effect (Olson et al., 2013), DHA regulates apoptotic pathways in various cells (de Urquiza et al., 2000). Although high dose DHA is an apoptosis enhancer in cancer cells (Olson et al., 2013), apoptosis inhibitory effect of DHA is detected in the neurons (Florent et al., 2006). NPD1, a neuroprotective derivative of DHA, is a potent inhibitor of oxidative stress-induced apoptosis and of cytokine-mediated pro-inflammatory induction of COX-2 in human retinal pigment epithelial (RPE) cells. It upregulates protective Bcl-2 proteins and inhibits pro-inflammatory gene expression (IL-1B, COX-2) (Bazan, 2006). Also, NPD1 protects the brain toward leukocyte infiltration, cytokine production and microglia activation (Hong et al., 2003; Laye et al., 2018; Lukiw et al., 2005). For this reason, the therapeutic effect of DHA in cancer types and neurodegenerative diseases such as PD is being investigated. In addition, there is no study showing the effect of DHA on expression of apelin and apelin receptor of CNS. Instead, the effect of DHA on the apelin gene expression was shown on aged and young fat cells (Prostek et al., 2016).

In our previous study, it has been demonstrated that DHA has neuroprotective effect against MPTP-induced PD (Ozkan et al., 2016). It is also known that DHA increases oxidative stress, although it has anti-inflammatory effect. However, the mechanism of protective effect of DHA against PD is not known. It is thought that the expression of a molecule which is altered by DHA may play a role in this neuroprotective effect.

Apelin was first isolated and characterized in 1998 from bovine stomach tissue extracts (Tatemoto et al., 1998). The 77-amino acid prepropeptide called preproapelin is expressed by the human apelin gene and can be cleaved by angiotensin-converting enzyme 2 (Kalantaripour et al., 2017). Apelin exhibits several biological active forms including apelin 13, apelin 17, apelin 36. Apelin-13 has more biological potency than apelin 17 and 36 forms. There is one type receptor of endogenous apelin (APJ), which is coupled to G-protein. Apelin and its receptor (APJ) are also detected in many regions of nervous system such as hipothalamus, cerebellum, amydala, striatum, subthalamic nucleus, cortex and brainstem (De Mota et al., 2000; Lee et al., 2000; Medhurst et al., 2003). Apelin and its receptor are expressed in neurons, oligodendrocytes and astrocytes (Choe et al., 2000). Previous studies indicated that apelin and apelin receptors are expressed in neuron of ventral tegmental area (VTA), SN, striatum and mesolimbic pathway which have also dopaminergic neurons (Haghparast et al., 2018; Lee et al., 2000). The changes in expression of apelin in aged animals were observed in a study (Rai et al., 2017). An another study reported that apelin and apelin receptor expression had changed in a neurodegenerative disease (Kalantaripour et al., 2016). 6−OHDA induced experimental PD model animals showed a significant impairment in cognition. Apelin 13 had decreased cognitive impairment in 6−OHDA treated animals (Haghparast et al., 2018). It has been demonstrated that apelin has neuroprotective effects through ERK/AKT phosphorylation mechanism (Ishimaru et al., 2017). In the previous studies, it has been demonstrated that apelin has anti-oxidant, anti-apoptotic and anti-inflammatory effects and inhibits NMDA receptor activation (Ishimaru et al., 2017). Apelin prevents the excessive ROS production, mitochondrial dysfunction and accumulation of intracytoplasmic Ca^{++} (Kalantaripour et al., 2017).

It is not known whether the distribution of apelin and apelin receptor changes in PD. Previous study indicated that the impairment of cognition in 6−OHDA induced PD model animals was improved by administration of apelin in lateral ventricule (Haghparast et al., 2018). But there is no study about the apelin and apelin receptor expressions in MPTP model of PD. It is the first study that shows the distribution of apelin and apelin receptor expressions in the experimental PD. Furthermore, the effect of DHA on the distribution of apelin and apelin receptor, which has been shown to be beneficial for PD is unknown. This study was performed to investigate the distribution of apelin and APJ receptor protein in the central nervous system of mice with MPTP-induced PD. In addition, DHA has neuroprotective effect on the apelin and its receptor.

2. Materials and methods

Male *C57BL6* mice (3 months-old and weighing 25–30 g) were obtained from Akdeniz University Animal Care Unit. The animals were housed four to five mice in stainless steel cages at standart conditions (24 ± 2 °C and 50 ± 5% humidity) with a 12 h light-dark cycle. All experimental protocols managed on mice were done in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University.

2.1. Experimental design and treatment

Four experimental groups each consisting of ten mice were formed: Control group (C), DHA treated group (DHA), MPTP injected group (Parkinson) and MPTP injected and DHA treated group (Parkinson + DHA). Pure docosahexanoic acid (Sigma chemical CO, St Louis, MO, USA) was dissolved in corn oil at a concentration of 0.046 M and administered to DHA treatment groups for 30 day (36 mg/kg/day) by gavage (Hacioglu et al., 2012; Ozkan et al., 2016; Ozsoy et al., 2011; Parlak et al., 2018; Tanriover et al., 2010). The other groups were given a similar volume of vehicle (corn oil) in order to eliminate the effects of daily gavage and vehicle. Food and water were provided ad libitum through the experiments. On the 23rd day of DHA treatment, mice in the Parkinson and Parkinson + DHA groups were given four intraperitoneal (i.p.) injections (20 x 4 mg/kg 2 h interval) of freshly prepared MPTP hydrochloride (M-0896, Sigma-Aldrich, St, Louis, MO,

USA) dissolved in saline (Jackson-Lewis and Przedborski, 2007; Ozkan et al., 2016). Control and DHA groups were given equivalent volume of saline (pH 7.4). All groups of mice continued to take their usual diets and DHA for an additional week after MPTP injection.

2.2. Assessment of behavioral activity

2.2.1. Pole test

Bradykinesia intensity of mice was evaluated by pole test. Pole test was performed on the 7th day after the last MPTP injection. For this test, mice were placed head upward on top of a rough-surfaced pole (8 mm in diameter and 50 cm in length) double wrapped with sticking plaster to prevent slipping. Mice were allowed to climb down the pole freely to the base of pole (pre-trial). Animals were tested for two or three times for habituation. On the testing day, the time was recorded for the mice to turn (T-turn) its head downwards (movement initiation) and to t-turn and climb down the entire length of the pole (T-total). Averages of the best three measurements were taken as results (Kobayashi et al., 1997; Ozkan et al., 2016; Parlak et al., 2018).

2.2.2. Locomotor activity test

Open field test have shown to provide measures of motor activity. Locomotor activity cage (May 9908 model Activity Monitoring System: Commat Ltd, Ankara, Turkey) was used to evaluate MPTP-induced motor deficit (Dekundy et al., 2006; Khaldy et al., 2003; Willis and Robertson, 2004). Clear plexiglas locomotor activity chambers (42 cm L x 42 cm W x 30 cm H) were equipped with infrared photocells. These chambers contain fifteen photocell emitter and detector pairs which were placed to opposite walls (2 cm above the chamber floor), and another 15 photocell pairs that were located 8 cm above the floor. After a computer system records the breaks of the photocell beams, the mice's position in the chamber was calculated by the software at 0.1 s sensitivity. The ambulatory activity means that the calculated location of mice was completely changed. For testing all parameters, one mouse was placed in the center of the open field cage and its activity was recorded for 5 min. Before placing another animal, the cages were cleaned with 10% ethanol to attenuate odors.

2.2.3. Rotarod test

The motor coordination and balance of mice were tested by using the rotarod test (Rozas et al., 1997, 1998). Mice were acclimated to the rotarod apparatus for three consecutive days. On testing day, mice were habituated to the testing room for 30 min. Then they were placed on a rotating rod (model 7650 Rota-rod, Ugo Basile, Collegeville, PA, USA) which has the speed of 5, 10, 20, 30 or 40 rpms for 5 min. The time that the mice fell off the rod was recorded and they were given 5 min breaks between each recordings. The time for falls were recorded as well.

2.3. Tissue processing

After the motor performance tests, mice were anesthetized with urethane (1 g/kg, i.p.) which is diluted in saline. Mice were perfused transcardially with heparinized saline and the brain tissues were removed immediately. For immunohistochemical staining, cerebrum, cerebellum and pons samples were fixed in 10% formalin (100 ml 37% formaldehyde, 900 ml distilled water, pH ~7) at room temperature for 24 h. Formalin was removed by several washings with tap water. This was followed by dehydration; immersion in 70%, 80%, 90% ethanol for 24 h each and 100% ethanol for 4 h. After dehydration, tissues were cleared in xylene and embedded in paraffin wax. The transverse sections were cut on the microtome. In addition, the tissues to be used for western blot technique were only perfused with heparinized saline.

2.4. Immunohistochemistry

Formalin-fixed samples submerged in paraffin were cut into 5 µm pieces and put on poly-l-lysine-coated slides. Following the process of deparaffinization, slides were boiled in citrate buffer (pH 6.0) for the period of 10 min to retrieve antigen and left for cooling for the period of 20 min at room temperature. Afterwards, pieces were submerged in 3% hydrogen peroxide for the period of 20 min in order to prevent endogenous peroxidase. Incubation of the slides was performed in a humidified chamber by using an UltraV block (Lab-vision, Fremont, CA, USA) for the period of 7 min at room temperature. The excess of serum was removed, and the incubation of the sections was performed using primary antibodies apelin (sc-33805; Santa Cruz Biotechnology, CA, USA) rabbit polyclonal antibody at 1:150 dilution; apelin receptor (APJR) (bs-2430R; Bioss, Woburn, MA, USA) rabbit polyclonal antibody at 1:150 dilution for a night in a humidified chamber. The primary antibody was substituted with the suitable serum or non-immune IgG at the identical dilutions with the specific antibodies, and therefore, negative controls were carried out. Phosphate buffered saline (PBS) was used to wash the pieces three times for the period of 5 min and following this, their incubation was performed using biotinylated secondary antibody (HRPLSAB-2 system, K0609; DakoCytomation, Glostrup, Denmark) for the period of 30 min and afterwards using peroxidase labeled streptavidin (HRP LSAB-2 system, K0609; DakoCytomation, Glostrup, Denmark) for the period of 30 min. Following this, PBS was used to wash the slides, and visualization of peroxidase activity was accomplished by using 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, St.Louis, MO, USA; D4418) for the period of 3–5 min. Hematoxylin was utilized for the counterstaining of theslides. Their dehydration was carried out, and they were placed in Kaiser's glycerin gelatin (OB514196; Merck, Whitehouse Station, NJ, USA) and investigated using Zeiss Axioplan 100 light microscope (Zeiss, Oberkochen, Germany), and their photographs were taken.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

Protein extraction and immunoblot analysis were performed as described previously (Acar et al., 2012). Tissue samples for each group were weighed and put into a homogenization buffer supplemented with complete protease inhibitor cocktail (Boehringer, Mannheim, Germany). After homogenization, samples were centrifuged at 10,000 *g* for 10 min. Supernatants were collected and stored at −70 °C. The protein concentration was determined by Lowry assay (Lowry et al., 1951) and 50 µg of protein was applied per lane. Samples were subjected to SDS polyacrylamide gel electrophoresis (30% acrylamide in 7.5% gel) at 100 V for approximately 1.5 h and were then transferred onto nitrocellulose membranes (Pharmacia, Kirkland, Quebec, Canada) in a buffer containing 0.2 mol/L glycine, 25 mmol/L Tris and 20% methanol overnight, at +4 °C, under 32 V and 65 A. The membranes were blocked for 1 h with 5% non-fat dry milk (BioRad Laboratories, Hercules, CA, USA) and 0.1% Tween 20 (Sigma-Aldrich, St Louis, MO, USA) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2–7.4) at 4 °C. Membranes were incubated with a 1:200 dilution of rabbit polyclonal anti-apelin antibody (#sc33805, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200 dilution of rabbit polyclonal anti-apelin receptor antibody (#bs2430-R, Bioss, Woburn, MA, USA) overnight. After the washing steps, the membranes were further incubated with peroxidase goat anti-rabbit IgG antibody (Vector Laboratories, PI-1000, CA, USA), diluted 1:2000, for 1 h at room temperature. Immunolabeling was visualized by using the chemiluminescence-based SuperSignal CL HRP Substrate System (Pierce, Rockford, IL, USA) prepared according to the manufacturer's instructions, and the membranes were exposed to Hyperfilm (Amersham, Bucks, UK; 28,906,837). Membranes were also labeled by identical protocol for binding of a 1:6000 dilution of an anti-mouse β-actin antibody (Abcam, Cambridge, UK; ab 6276-100) as an internal control to confirm the equal loading of the samples. The bands were quantified using NIH image analysis software (Image J Version 1.36b, National Institutes of Health, USA).

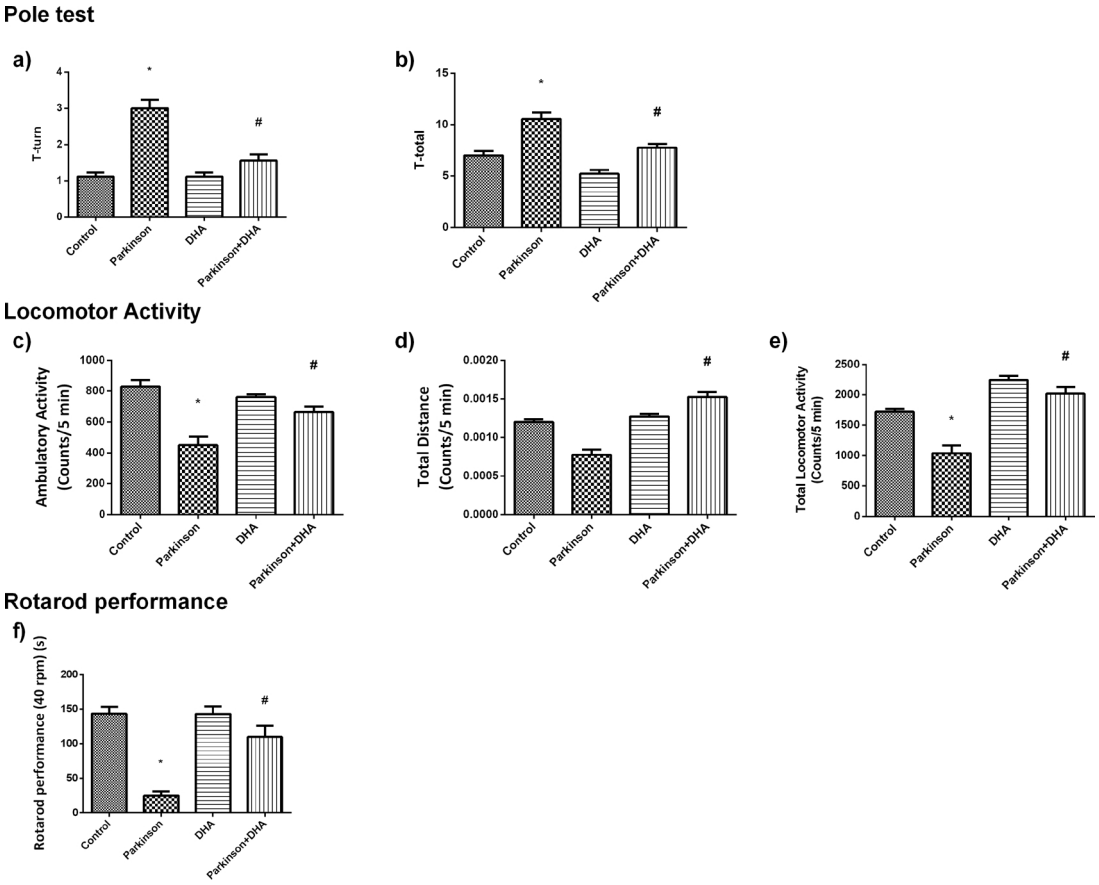


Fig. 1. Determination of bradykinesia by pole test. (a) T-turn (s). Data are means ± SEM. Statistical analyses are Kruskal Wallis followed by Dunn's multiple comparison test against indicated group. *p < 0.001 vs Control; #p < 0.05 vs Parkinson group. (b) T-total (s). Data are means ± SEM. Statistical analyses are One-way ANOVA followed by Tukey's multiple comparison test against indicated group. *p < 0.0001 vs Control; #p < 0.001 vs Parkinson group (n = 9).The effect of DHA on locomotor activities: (c) Ambulatory activity. Data are means ± SEM. Statistical analyses are One-way ANOVA followed by Tukey's multiple comparison test against indicated group. *p < 0.0001 vs Control; #p < 0.01 vs Parkinson group. (d) Total distance. Data are means ± SEM. Statistical analyses are Kruskal Wallis followed by Dunn's multiple comparison test against indicated group. #p < 0.0001 vs Parkinson group. (e) Total locomotor activity. Data are means ± SEM. Statistical analyses are One-way ANOVA followed by Tukey's multiple comparison test against indicated group. *p < 0.0001 vs Control; #p < 0.0001 vs Parkinson group (n = 9). (f) Rotarod performance at 40 rpm 7 days after the MPTP injection. Data are means ± SEM. Statistical analyses are Kruskal Wallis followed by Dunn's multiple comparison test against indicated group. *p < 0.001 vs Control; #p < 0.05 vs Parkinson group (n = 9).

2.6. Statistical analysis

Motor activity tests, apelin and apelin receptor Western blotting band values of cerebrum, cerebellum and pons samples of Control, Parkinson, DHA and Parkinson + DHA groups were analyzed by ANOVA followed by post-hoc Tukey tests for suit with normal distribution and Kruskal Wallis followed up Mann Whitney U test or Dunn's multiple comparison test for not suit with normal distribution. Probability values of less than 0.05 were considered significant. The data were analysed with SPSS version 13.0 software (Chicago, IL, USA).

3. Results

3.1. Motor performances

Changes in bradykinesia are shown in Fig. 1a and b. Time required to turn (T-Turn) (sn) and time required to t-turn + climb down the entire length of the pole (T-total) (sn) were determined for all groups. MPTP injected mice showed marked bradykinesia in the pole test compared with the control and DHA groups. T-Turn and T-Total time had improved in the MPTP + DHA group compared with the MPTP group.Basal locomotion tests were performed in automated locomotor chambers. Horizontal and vertical activities did not reveal any difference between all groups (data not shown). Ambulatory activity and total locomotor activity were decreased in the MPTP group compared with the control group. DHA treatment increased the ambulatory activity, total distance and total locomotor activity of the MPTP + DHA group compared with the MPTP group (Fig. 1c, d and e).The time mice stayed on the rod at 40 rpm was decreased in the MPTP group compared to the control group. Motor coordination (40 rpm) in the MPTP + DHA group was significantly better than the MPTP group (Fig. 1f).

3.2. Immunohistochemical findings

3.2.1. Apelin immunolocalization in the cerebrums of control, Parkinson, DHA and Parkinson + DHA groups

The apelin immunoreactivity was detected in cerebrum of Control, Parkinson, DHA and Parkinson + DHA groups. In all groups, strong apelin immunoreactivity was observed and there was no difference between the groups (Fig. 2).

3.2.2. Apelin immunolocalization in the cerebellums of control, Parkinson, DHA and Parkinson + DHA groups

The immunoreactivity of apelin in cerebellum was very strong in Control and Parkinson group; whereas the apelin expression was decreased in DHA and Parkinson + DHA groups (Fig. 3).

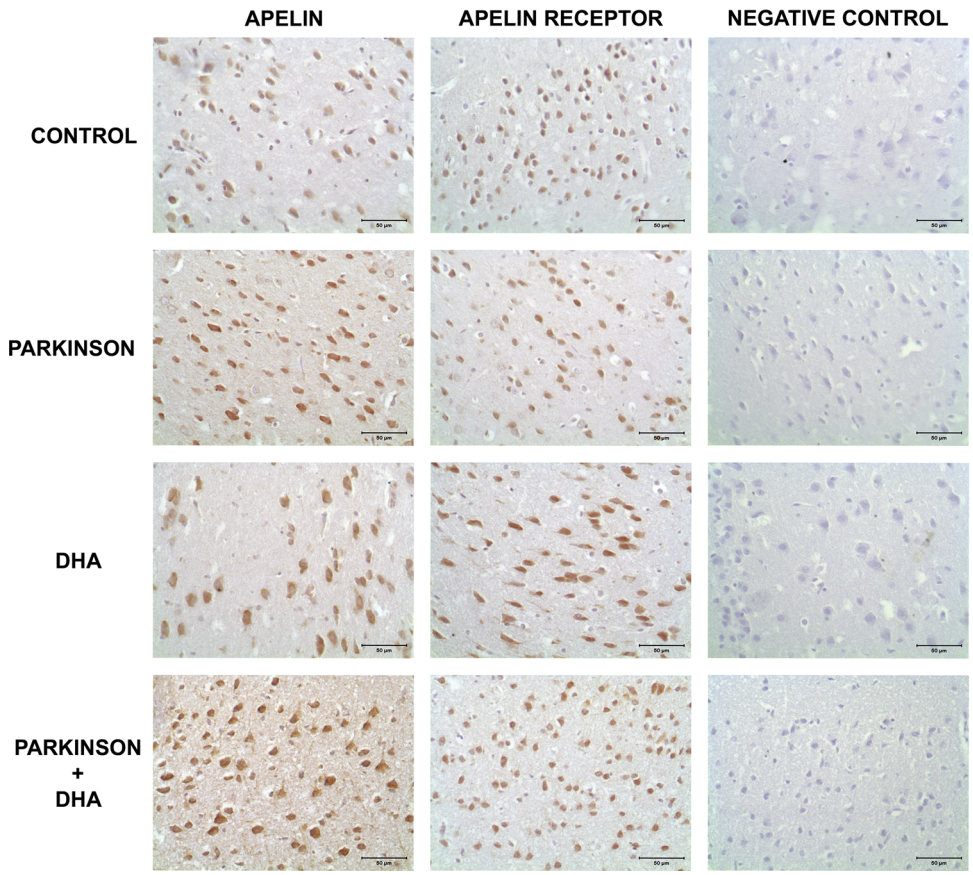


Fig. 2. Apelin and apelin receptor immunohistochemistry in cerebrums of Control, Parkinson, DHA and Parkinson + DHA groups. Negative controls of each group are also shown. Scala bars: 50 µm.

3.2.3. Apelin immunolocalization in the pons of control, Parkinson, DHA and Parkinson + DHA groups

The immunoreactivity of apelin was very strong in the Control group compared to other groups in pons **(**Fig. 4**).**

3.2.4. Apelin receptor immunolocalization in cerebrums of control, Parkinson, DHA and Parkinson + DHA groups

The apelin receptor immunohistochemistry was performed in cerebrums of Control, Parkinson, DHA and Parkinson + DHA groups. All groups showed similar apelin receptor immunoreactivity (Fig. 2**)**.

3.2.5. Apelin receptor immunolocalization in cerebellums of control, Parkinson, DHA and Parkinson + DHA groups

The apelin receptor immunoreactivity was detected in cerebellums of Control, Parkinson, DHA and Parkinson + DHA groups. It was strong positive in all groups (Fig. 3).

3.2.6. Apelin receptor immunolocalization in the pons of control, Parkinson, DHA and Parkinson + DHA groups

Apelin receptor immunohistochemistry was performed in pons of Control, Parkinson, DHA and Parkinson + DHA groups. While the control group had very strong positive apelin receptor expression, the Parkinson, DHA and Parkinson + DHA groups had strong positive apelin receptor expression in their pons (Fig. 4).

3.3. Western blotting findings

The western blot quantification was performed via NIH image analysis software (Image *J* Version 1.36b, National Institutes of Health, USA) on Western blot bands for apelin and apelin receptor expressions of Control, Parkinson, DHA and Parkinson + DHA groups and got numerical values.

3.3.1. Apelin expression in the cerebrums of control, Parkinson, DHA and Parkinson + DHA groups

According to western blotting results, apelin expressions in cerebrums were 0.7597 ± 0.07202, 0.7744 ± 0.09370, 0.7970 ± 0.1365, 0.6362 ± 0.1268 in Control, Parkinson, DHA and Parkinson + DHA groups, respectively. There was no statistically significant difference between the groups (Fig. 5).

3.3.2. Apelin expression in the cerebellums of control, Parkinson, DHA and Parkinson + DHA groups

The apelin expressions in cerebellums of DHA and Parkinson + DHA groups were lower compared to the Control and Parkinson groups. But the difference wasn't statistically significant. Numerical values of western blot bands were 0.9182 ± 0.2106, 1.035 ± 0.1858, 0.5499 ± 0.1819, 0.5098 ± 0.1624 in the Control, Parkinson, DHA and Parkinson + DHA groups, respectively (Fig. 6).

3.3.3. Apelin expression in pons of control, Parkinson, DHA and Parkinson + DHA groups

According to our western blotting results, apelin expressions in pons of Parkinson, DHA and Parkinson + DHA groups were lower compared to the control group. Numerical values of western blot bands were 1.144 ± 0.5418, 0.5798 ± 0.1997, 0.4525 ± 0.1449, 0.4911 ± 0.1581 in Control, Parkinson, DHA and Parkinson + DHA groups, respectively. The difference between Control and Parkinson (p = 00,470), Control and DHA (p = 00,116), Control and Parkinson + DHA (p = 00,172) groups were statistically significant (Fig. 7).

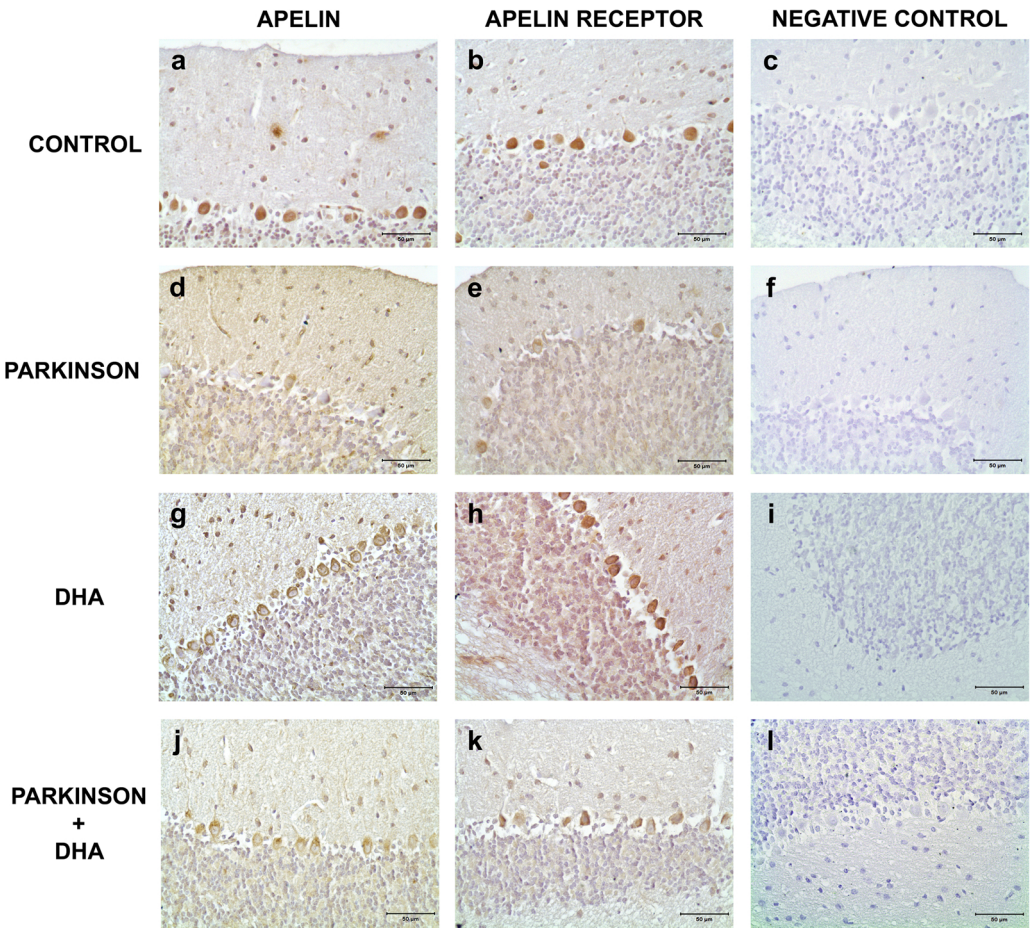


Fig. 3. Immunohistochemistry on cerebellums of Control, Parkinson, DHA and Parkinson + DHA groups for apelin (a, d, g and j) and apelin receptor (b, e, h and k) are shown. Negative controls are shown on the last column. Scala bars: 50 µm.

3.3.4. Apelin receptor expression in the cerebrums of control, Parkinson, DHA and Parkinson + DHA groups

Theapelin receptor expressions in cerebrums were 1.136 ± 0.1337, 1.026 ± 0.1283, 0.9560 ± 0.1310, 0.8604 ± 0.07002 in Control, Parkinson, DHA and Parkinson + DHA groups, respectively. There was no statistically significant difference between the groups (Fig. 8).

3.3.5. Apelin receptor expression in the cerebellums of control, Parkinson, DHA and Parkinson + DHA groups

According to our western blotting results, apelin receptor expressions in cerebellums were 1.057 ± 0.1449, 0.9356 ± 0.1520, 0.9303 ± 0.09422, 1.031 ± 0.02679 in Control, Parkinson, DHA and Parkinson + DHA groups, respectively. The difference between Control and Parkinson + DHA ($p = 0.0021$), Parkinson and Parkinson + DHA ($p = 0.0017$), DHA and Parkinson + DHA ($p = 0.0153$) groups were statistically significant (Fig. 9).

3.3.6. Apelin receptor Western expression in pons of control, Parkinson, DHA and Parkinson + DHA groups

The apelin receptor expressions in pons of Parkinson, DHA and Parkinson + DHA groups were lower compared to the Control group. Numerical values of western blot bands were 1.816 ± 0.2877, 1.237 ± 0.09432, 1.263 ± 0.06892, 1.406 ± 0.09251 in Control, Parkinson, DHA and Parkinson + DHA groups, respectively. The difference between Control and Parkinson ($p = 0.0453$), Control and DHA ($p = 0.0170$), Control and Parkinson + DHA ($p = 0.0418$) groups were statistically significant (Fig. 10).

4. Discussion

In the present study, the effect of DHA treatment on apelin and its receptor in the experimental model of PD via MPTP injection has been investigated and this effect has been evaluated by western blot and immunohistochemical parameters. PD is a complex disease and the etiology of PD is not clearly known yet. However, it could be explained by the environmental and genetic factors. The neurotoxins such as hydroxylated analogue toxin of 6−OHDA, paraquate, rotenone and MPTP are used to create experimental PD. MPTP is the most important and most frequently used neurotoxin in the animal models of PD. This toxin is mainly used in primates, mice and rats. *C57BL6* mice strain was found to be significantly more selective to a systemic injection of MPTP than the other mice race. MPTP can pass through the blood-brain barrier and is converted to MPP + by MAO-B enzymes which present in astrocytes. MPTP could have been given in a variety of type such as gavage or stereotaxic injection. However in this study, MPTP was intraperitoneally administrated at four doses consistent with literature (4 x 20 mg/kg) in every two hours (Beal et al., 1998; Date et al., 1990).

The main fatty acids in the brain are EPA and DHA (Crawford, 1993). It is known that DHA is reduced in neurodegenerative diseases like PD (Youdim et al., 2000). It has been shown that DHA, which is important for brain functions, changes the fatty acids in the brain's neuron membranes when it is given as a supplement (Kidd, 2000; Ogawa et al., 1987; Youdim et al., 2000).DHAH, a hydroxylated derivative of DHA, showed a protective effect by increasing the number of TH + cells in the PD model (Hernando et al., 2018). In addition, the administration of this derivative reduced AB oligomers and tau phosphorylation in Alzheimer Disease model (Torres et al., 2014). DHA and

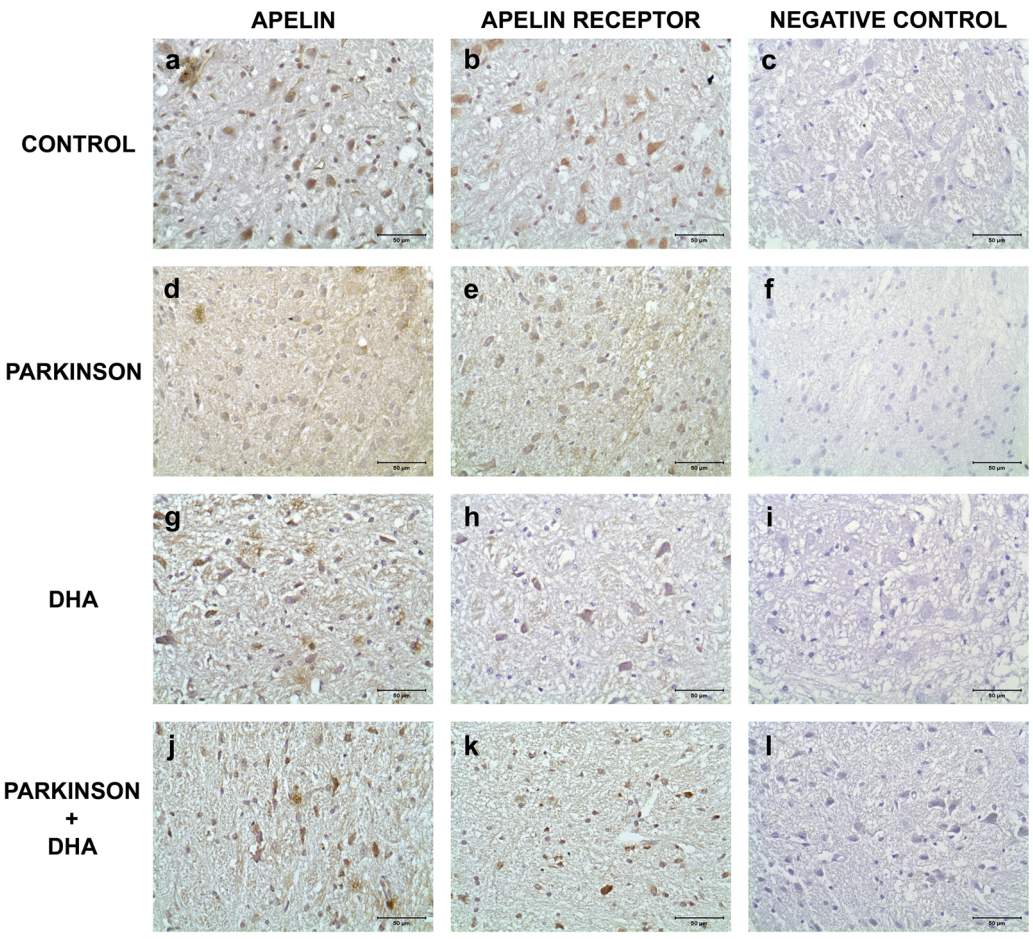


Fig. 4. Apelin immunohistochemistry in pons of Control (a), Parkinson (d), DHA (g) and Parkinson + DHA groups (j). Apelin receptor immunohistochemistry in pons of Control (b), Parkinson (e), DHA (h) and Parkinson + DHA groups (k). Negative controls are also shown. Scala bars: 50 µm.

DHAH supplementation decreased the number of astroglia and microglia or neuroinflammation (Hernando et al., 2018). n-3 PUFA inhibited the effects mediated by proresolving factors IL-1β and TNF-α, down-regulating of several enzymes of arachidonic acid cascade in CNS or membrane fluidity and therefore inhibiting membrane ion channel (Bazinet and Laye, 2014; Sun et al., 2018). Lin et al reported that chronic n-6 PUFA deprivation diminished the loss of AA and increased the rate of loss of DHA in rat brain phospholipids (Lin et al., 2015). n-6 PUFA deprivation reduces expression of AA-selective enzymes [(cytosolic phospholipase A_2 ($cPLA_2$) and COX-2)] and increases expression of DHA-selective enzymes [calcium-independent phospholipase A_2 ($iPLA_2$) and 15-lipoxygenase (LOX)] (Kim et al., 2011). DHA is used at various concentrations in studies (10–100 mg/kg/day). In this study, the DHA dose was selected according to previous studies (Hacioglu et al., 2012; Ozkan et al., 2016; Ozsoy et al., 2011; Tanriover et al., 2010).

Motor coordination, balance and locomotor activity are known to be impaired in the experimental PD models. To determine the locomotor activity, the test was performed on the 7^{th} day following MPTP injection. The number of steps taken by the animals for 5 min is detected with photocells and recorded with an automatic device. The locomotor activity of mice was estimated as ambulatory, total distance and total locomotor activity. The present findings are in agreement with the results from previous studies which demonstrate the impairment of motor activity in the experimental PD model. On the other hand, the administration of DHA improved impairment of locomotor activity in the Parkinson group. The results of the pole test indicating the intensity of the bradykinesia showed that the T-turn and the T-total times were increased in the Parkinson group, while DHA treatment significantly shortened both parameters (Ozkan et al., 2016; Ozsoy et al., 2011). The rotarod test has been used for determination of motor coordination and balance. The length of stay of the animals on the rotating shaft, turning at a constant speed of 5, 10, 20, 30, 40 rpm has been determined. In the Parkinson group, the duration of stay on the bar was shorter than in the control groups which is in accordance with literature (Rozas et al., 1998). Compared to the Parkinson group, the difference was found in the DHA treated Parkinson group. As a result, the administration of DHA was shown to have beneficial effect on motor coordination.

Apelin and its receptor were determined in cerebrum, cerebellum and pons. Previous studies have shown that apelin presents in hypothalamus, subthalamic nucleus, amygdala, cerebellum and hippocampus which is in agreement with our results (Reaux et al., 2002). The experimental PD model was utilised by MPTP administration. Apelin and its receptor were not changed in cerebrum whereas its receptor protein of cerebellum was decreased in the Parkinson + DHA group. PD patients have significantly more cerebellar atrophy and atrophy pattern predominantly involving cerebellar lobules VI and VII (Schreglmann et al., 2017). The other study indicated that the cerebellum has important dual task. Functional MRI shows the role of cerebellum in performing motor and cognitive task in PD (Gao et al., 2017). It has been suggested that PD is a multisystem atrophy (Kalita et al., 2017). PD patients often suffer from cognitive dysfunction. Apelin-13 significantly attenuates the cognitive impairment in 6−OHDA-treated animals (Haghparast et al., 2018). In addition, another article has shown that PD dystonia can be involved in cerebello basal ganglia communication (Akbar and Kim, 2002). PD dystonia is associated with cerebellar abnormalities. This disorder is primarily associated with the pathology of the cerebellum that dystonia can orginate in cerebellar

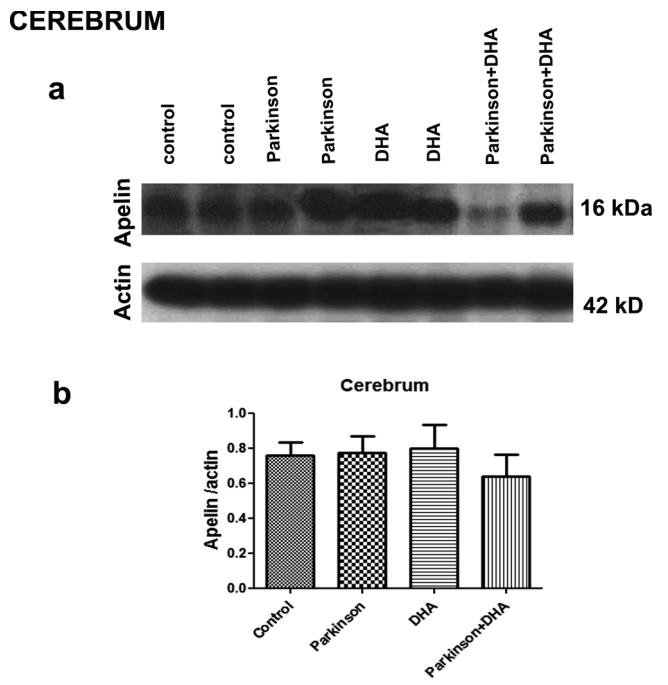


Fig. 5. a) Western blotting results of apelin in cerebrums of Control, Parkinson, DHA and Parkinson + DHA groups. Beta-actin was used as an internal control. b) Quantitative analysis of apelin Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The image is representative of three independent experiments.

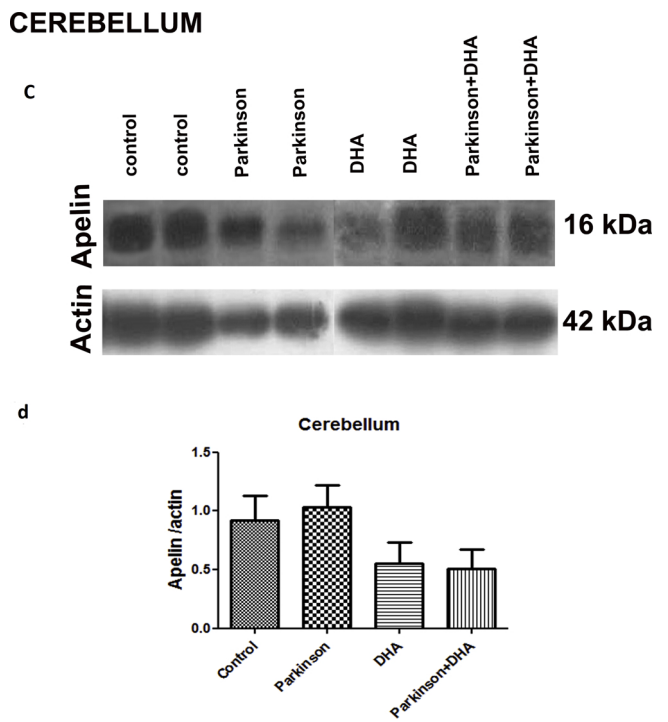


Fig. 6. a) Western blotting results of apelin in cerebellums of Control, Parkinson, DHA and Parkinson + DHA groups. Beta-actin was used as an internal control. b) Quantitative analysis of apelin Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The image is representative of three independent experiments.

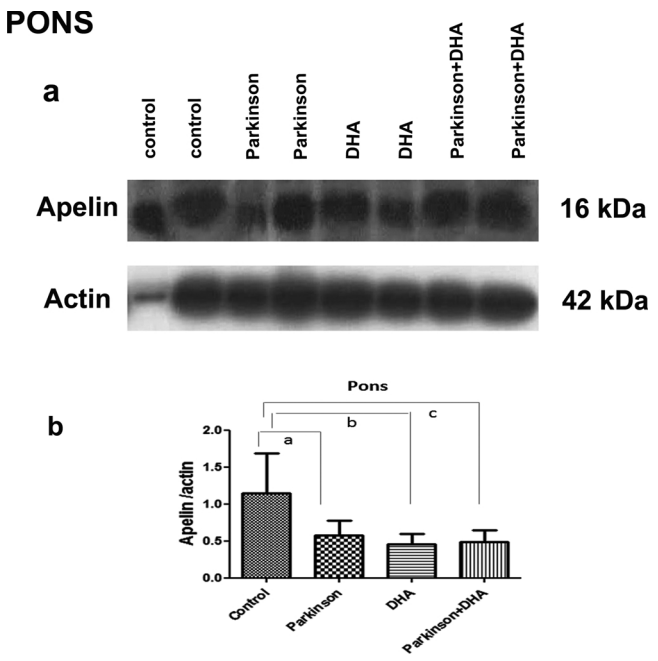


Fig. 7. a) Western blotting results of apelin in pons of Control, Parkinson, DHA and Parkinson + DHA groups. Beta-actin was used as an internal control. b) Quantitative analysis of apelin Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The image is representative of three independent experiments.

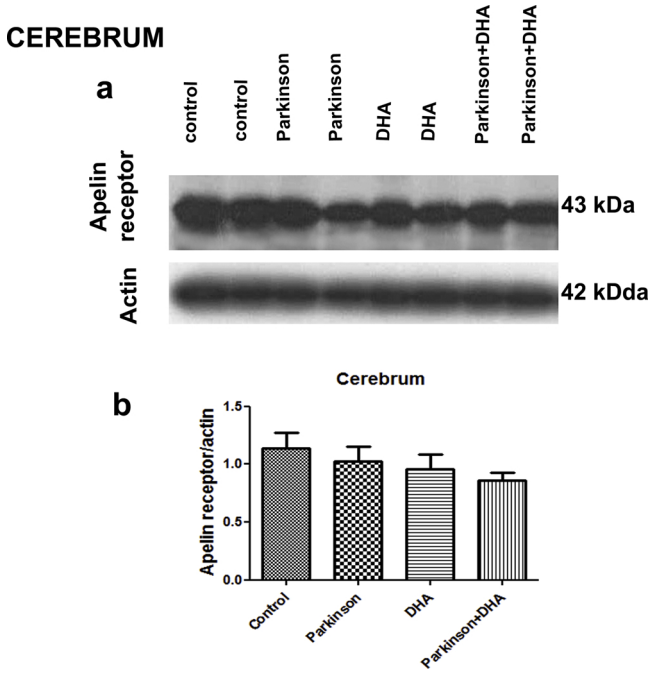


Fig. 8. a) Western blotting results of apelin receptor in cerebrums of Control, Parkinson, DHA and Parkinson + DHA groups. Beta-actin was used as an internal control. b) Quantitative analysis of apelin receptor Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The image is representative of three independent experiments.

cells. Recent optogenetic studies indicated that underlying mechanism of dystonia is the abnormal bursting potential of cerebellar purkinje cells and cerebellar nuclei. This abnormal burst firing may be responsible for cerebellar dystonia (Tewari et al., 2017). MPTP has been

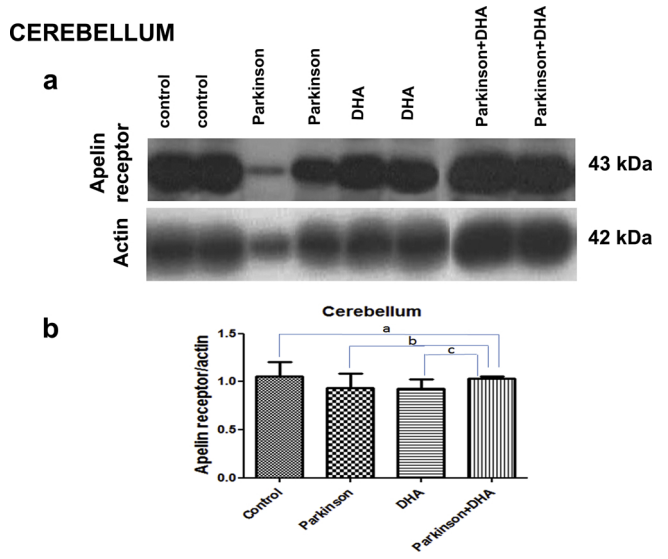


Fig. 9. a) Western blotting results of apelin receptor in cerebellums of Control, Parkinson, DHA and Parkinson + DHA groups. Beta-actin was used as an internal control. b) Quantitative analysis of apelin receptor Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The image is representative of three independent experiments.

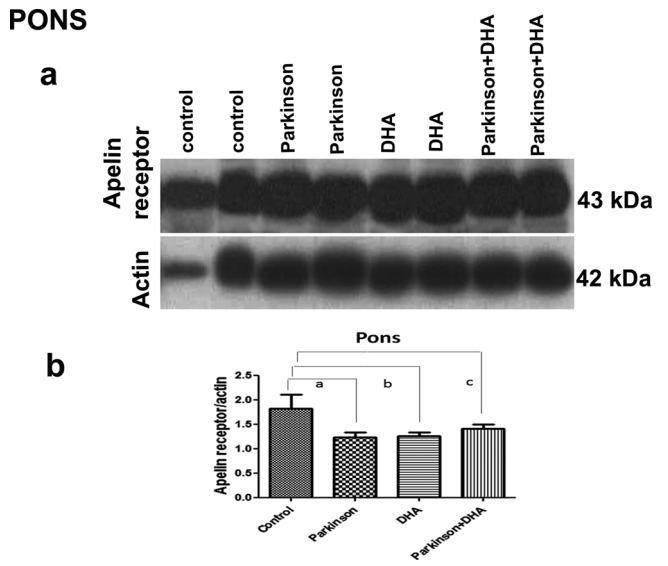


Fig. 10. a) Western blotting results of apelin receptor in pons of Control, Parkinson, DHA and Parkinson + DHA groups. Beta-actin was used as an internal control. b) Quantitative analysis of apelin receptor Western blotting data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding beta-actin intensity value. The image is representative of three independent experiments.

found to reduce the apelin and its receptor protein levels in pons. Previous studies have shown that noradrenergic neurons of locus coeruleus are degenerated in PD. The degree of neuronal loss in locus coeruleus was even greater and earlier than that of nigral dopaminergic neurons (Yao et al., 2015; Zarow et al., 2003). It is noteworthy that depression rate is increased in PD patients. Depression in PD patients can be due to the neurodegeneration of serotonin secreting neurons in raphe nucleus (Faggiani et al., 2015) There is a relationship between depression and serotonin. This can be explained by sleeping disorders of PD patients. MPTP is known to cause neuronal death by producing mitochondrial insufficiency and oxidative stress. PD causes neurodegeneration with the effect of some cytokines secreted from microglia in pons. It is known that microglia activation leads to the production of ROS, TNF-α IL-6 and COX-2 whereas antioxidant enzymes such as SOD and GSH decrease in pons in PD (Yao et al., 2015). There is a relationship between the loss of neurons and microglia activation in pons. There are also multifactorial relationships between the etiopathology of PD (Yao et al., 2015). Previous study has shown that the serotonin and norepineprine levels were decreased in locus ceruleus and dorsal raphe nucleus of experimental PD. This decrease may be related to the apelin and apelin receptor because apelin is known to have anti-inflammatory, anti-apoptotic and antioxidant properties. This properties causessto microglia inhibition (Gu et al., 2013; Pouresmaeili-Babaki et al., 2018). Apelin and its receptor downregulation of pons may be explained by these features.

DHA seems to have an effect on the expression of apelin and its receptor protein in cerebellum and pons while there is no effect in cerebrum. The knowledge about the relationship between n-3 fatty acids and lipid peroxidation is very sparse and contradictory. Nenseter and Drevon reported that n-3 fatty acid intake leads to increased lipid peroxidation, which may enhance the unsaturation index (Nenseter and Drevon, 1996). According to this hypothesis, an enhancement in the number of double bounds increases the fatty acid oxidation (Liu et al., 2017). This hypothesis may be true for *in vitro* studies but is rather complex in *in vivo* studies (Mori et al., 2003). Consequently, the effects of fatty acids on lipid peroxidation and oxidative stress are contradictory. Song and Miyazawa demonstrated that lipid peroxidation products increased together with an increase in polyunsaturation (Song and Miyazawa, 2001). On the other hand, studies have shown that DHA application significantly inhibits TBARS production (Glozman et al., 1998; Mori et al., 2003; Sarsilmaz et al., 2003). These inconsistencies may be related to differences in the populations studied, the quantity and presentation of the n-3 PUFAs, the duration of the study, or the antioxidant content of the supplement or background diet. It has also been suggested that the total concentration of polyunsaturate products, rather than the unsaturation index, may be a more important factor affecting lipid peroxidation (Brude et al., 1997). Regarding our findings, supplementary DHA protects brain and retina tissues from harmful effects of oxidative stress. Previous study has shown that DHA enhances gen transcription of apelin by acting on nuclear receptors or retinoid receptor (de Urquiza et al., 2000). It has been shown that DHA affects apelin gene expression in adipose tissue culture cell. While DHA had reduced apelin expression in mature cells, there was no effect on the cells incubated for different durations (Prostek et al., 2016). Given these studies, it can be expected that DHA may have different effects on different tissues.

In conclusion, this study demonstrates an improvement of locomotor activity parameter via DHA supplementation in MPTP toxicity. The differences in the expression of apelin and its receptor in pons and cerebellum may be related to different neurons and secreted different neurotransmitters. Further studies are required to clarify these aspect.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

This work was supported by the Akdeniz University research fund.

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