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The effects of docosahexaenoic acid on glial derived neurotrophic factor and neurturin in bilateral rat model of Parkinson's disease

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Abstract: Parkinson's disease (PD) is the second most common neurodegenerative disorder marked by cell death in the *Substantia nigra* (SN). Docosahexaenoic acid (DHA) is the major polyunsaturated fatty acid (PUFA) in the phospholipid fraction of the brain and is required for normal cellular function. Glial cell line derived neurotrophic factor (GDNF) and neurturin (NTN) are very potent trophic factors for PD. The aim of the study was to evaluate the neuroprotective effects of GDNF and NTN by investigating their immunostaining levels after administration of DHA in a model of PD. For this reason we hypothesized that DHA administration of PD might alter GDNF, NTN expression in SN. MPTP neurotoxin that induces dopaminergic neurodegeneration was used to create the experimental Parkinsonism model. Rats were divided into; control, DHA-treated (DHA), MPTP-induced (MPTP), MPTP-induced+DHA-treated (MPTP+DHA) groups. Dopaminergic neuron numbers were clearly decreased in MPTP, but showed an increase in MPTP+DHA group. As a result of this, DHA administration protected dopaminergic neurons as shown by tyrosine hydroxylase immunohistochemistry. In the MPTP+DHA group, GDNF, NTN immunoreactions in dopaminergic neurons were higher than that of the MPTP group. In conclusion, the characterization of GDNF and NTN will certainly help elucidate the mechanism of DHA action, and lead to better strategies for the use of DHA to treat neurodegenerative diseases.

Key words: Parkinson's disease, substantia nigra, DHA, GDNF, NTN

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. Neurodegenerative disorders are characterized by a progressive and specific loss of neurons. PD results from the progressive degeneration of dopamine neurons that innervate the striatum [1]. Alteration of the postural reflex, bradykinesia, muscle rigidity and resting tremor are clinical symptoms that characterize this disease [2]. Furthermore, clinical symptoms appear after 50-60% of neuronal loss, cell death and degeneration in the *Substantia nigra* (SN) [3]. Since age is a consistent risk factor, an age-dependent cumulative insult mechanism may be responsible for the selective degeneration of nigrostriatal neurons. Moreover, the increase of free radicals with age is the reason for the decrease in concentration of the polyunsaturated fatty acid (PUFA) in the cell membrane. Supplementation of the diet with these PUFAs may help to delay changes associated with neurodegenerative diseases such as PD [4].

Docosahexaenoic acid (DHA) is the major PUFA in the phospholipids fraction of the brain and is required for normal neuronal function [5]. Maintaining concentrations of this PUFA is essential for enhanced cognitive, learning and memory functions. Neurodegenerative disorders such as PD, often exhibit significant declines in DHA and other PUFAs, which may in part, contribute to some of the observed declines in brain functions [6]. Considerable effort has been devoted to the search for molecules that might exert trophic influences on midbrain dopamine neurons, and potentially be of therapeutic value in the treatment of PD.

Glial cell line-derived neurotrophic factor (GDNF) was originally identified as a survival factor for mid-

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brain dopaminergic neurons [7]. The members of the GDNF family is composed of GDNF, neurturin (NTN), persephin (PSP), and artemin (ART) [7], of which GDNF and NTN are responsible for the development and survival of the enteric neurons, and NTN for parasympathetic neurons [8]. GDNF promotes recovery of the injured nigrostriatal dopaminergic system and improves motor functions in rodent and non-human primate models of PD [8]. GDNF and NTN are very potent trophic factors [9] and they have been shown to exert neuroprotective effects on lesioned nigral dopaminergic neurons for PD [8].

The aim of the study in light of this knowledge was to evaluate the neuroprotective effects of GDNF and NTN by investigating their immunolocalization after administration of DHA to a rat model of PD. For this reason we hypothesized that DHA administered to a rat model of PD might alter GDNF and NTN expression in SN.

Materials and methods

Animals. A total of 24 adult Wistar male rats (380-420 g) were housed at 22-24°C under controlled conditions with free access to standard rat chow and water. The experimental protocol was approved by the animal care and usage comittee of Akdeniz University and was in accordance with the Declaration of Helsinki and International Association for the Study of Pain Guidelines.

Experimental protocol. In this study 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) neurotoxin that induces dopaminergic neurodegeneration was used to create a model of Parkinson's disease. Significantly, this model closely mimics the clinical symptoms found in PD that is more closely related to human Parkinsonism.

The rats were randomly divided into 4 groups as follows: (1) Control, (2) DHA-treated (DHA), (3) MPTP-induced (MPTP), (4) MPTP induced+DHA treated (MPTP+DHA). DHA (D2534, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in corn oil at a concentration of 0.046 M and was given to the treatment groups for 30 days (36 mg/kg/day) by gavage [10]. In order to eliminate the effects of daily gavage and vehicle; other groups received a similar volume of corn oil alone. Three weeks after gavage procedure, MPTP and MPTP+DHA animals were anesthetized with 400 mg/kg chloral hydrate (K91627425, Merck KGaA, Darmstadt, Germany) intraperitoneally. MPTP (M-0896, Sigma-Aldrich, St. Louis, Mo,USA) (100 g/1 l saline) was infused bilaterally into the medial forebrain bundle using a Hamilton microsyringe at a rate of 0.33 l/min [11], according to the following coordinates adapted from the Pellegrino's atlas [12]: anteroposterior (AP) 2.2 mm from the bregma, mediolateral (ML) ±1.5 mm from midline and dorsoventral (DV) 8.0 mm from the skull. After surgery, animals were allowed to recover from anesthesia in a temperature controlled chamber and then placed in individual cages. The observers were blind to the type and source of rat/tissue examined.

Tests of motor activity. Seven days after the creation of the experimental PD model, motor activity of the rats was investigated using the "vertical pole" and "vertical wire" tests. The results of these test confirmed that the created model of PD was reliable.

For the vertical pole test, the animal was placed face up on a cloth-tape-covered pole (3.0 cm diameter, 150 cm length), which was held in a horizontal position, then the pole was gradually lifted to a vertical position and the time a rat stayed on the pole was recorded for a maximum of 120 s. In this test, the animal with deficits in motor coordination and balance will fall off the pole [13].

We also analyzed the rat catalepsy state on vertical wire netting (size 56.5 × 23.5 cm; mesh 1 × 1 cm; wire diameter 2 mm). The rats were placed with all paws on the wire net and the time taken for at least one paw to be actively displaced from the bar (descent latency) was determined [14].

Tissue collection. At the end of the treatment period and physiological tests, rats were anesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.), perfused transcardially with heparinized saline and brains were removed immediately. The samples were fixed immediately in formalin at room temperature for 8 h. dehydrated in ascending ethanol series, and embedded in paraffin for immunohistochemical analysis. 5-µm thick sections were collected onto poly-l-lysine-coated slides (Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry. We used tyrosine hydroxylase (TH) immunoreactivity to mark dopaminergic neurons in SN of PD model. For GDNF, NTN and TH immunohistochemistry, paraffin sections were deparaffinized, rehydrated and blocked for endogenous peroxidase activity with methanol containing 3% H2O2 for 15 min and for nonspecific binding with universal blocking reagent (BioGenex, San Ramon, CA, USA) for 10 min at room temperature [15]. Anti-rabbit GDNF (sc #9010), anti-goat NTN (sc #8173) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in dilution buffer (1/250) or mouse anti-TH (Calbiochem, CA, USA #657010) (1/100) were applied for 1h at room temperature in a humidified chamber. For negative controls the primary antibodies were replaced by appropriate isotype antibodies at the same concentration. After several washing steps in PBS, sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated horse anti-goat IgG secondary antibody (1/400 dilution Vector Lab. Burlingame, CA, USA) for 30 min followed by LSAB streptavidin-peroxidase complex (Dako, Carpinteria, CA, USA) incubation for 30 min and were rinsed with PBS. Antibody-antigen complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (BioGenex). Sections were counterstained with Mayer's hematoxylin (Dako), dehydrated, mounted and examined by a Zeiss-Axioplan (Oberkochen, Germany) microscope.

Evaluation of tyrosine hydroxylase (TH)-positive neurons. To assay changes in the number of dopaminergic neurons in the SN, the total numbers of TH-stained neurons were counted independently by two observers blinded to the type and source of the tissues under a light microscope (40X magnification) in six slides from each of the groups. The intra-individual and inter-individual coefficients of variation were 7 and 10%, respectively, for the evaluation. The average of counts was presented.

Semi-quantitative analysis of staining intensities. The intensity for TH, GDNF and NTN immunoreactivity was semi-quantitatively evaluated using the following intensity categories: no staining (-), weak but detectable staining (+), moderate or distinct staining (++), strong or intense staining (+++). The data are presented in Table 1.

Statistical analysis. Differences in motor behavior among groups were compared by non-parametric Kruskal-Wallis test, followed by post hoc Mann-Whitney U test. The data from TH-positive neuron counts were normally distributed as tested by Kolmogorov-Smirnoff test and therefore, were analyzed with Student's t-test or one-way ANOVA, followed by post hoc Holm-Sidak test when appropriate. All statistical analyses were performed using Sigmastat for Windows, version 3.0 (Jandel Scientific Corporation, San Rafael, CA). Data are presented as the mean ± SEM. Differences were considered to be significant at $p<0.05$.

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Table 1. Semi-quantitative evaluation of immunostaining intensities. Staining intensity categories: (-)no staining, (+)weak but detectable staining, (++) moderate or distinct staining, (+++) strong or intense staining.

Staining intensity	Control	DHA	MPTP	MPTP+DHA
TH	+++	+++	+++	+++
GDNF	+++	+++	+	++
NTN	++	++	+	++

Table 2. Representative table presents tyrosine hydroxylase immunopositive dopaminergic neuron numbers

	Control	DHA	MPTP	MPTP+DHA
TH (+) Neuron Number	399±3	442±1.5	152±2[a]	309±2.4[a,b]

The data are presented as Mean±SEM. [a] MPTP group was significantly lower than that of control and DHA groups ($p<0.05$). [b] MPTP+DHA group was significantly higher than that of MPTP group ($p<0.05$).

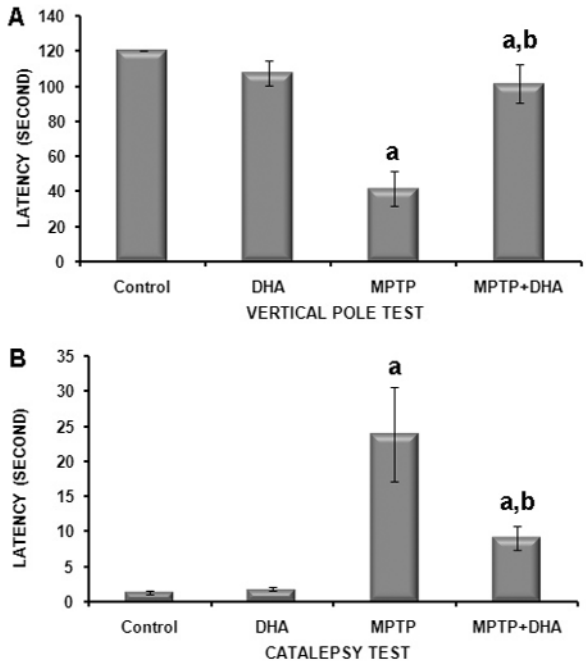

Fig. 1. The comparison of motor activities using vertical pole test (**A**); vertical wire test (**B**). C; Control, D; DHA group, MPTP, MPTP+DHA group. Data are expressed as means ± S.E. A. P<0,05 vs control group, B. P<0.05 vs MPTP group.

Results

Motor activity

A significant ($p<0.05$) decrease in motor activity was found in the groups with experimental Parkinsonism when compared to the control (Fig. 1A, B). In the vertical pole test, control (120 sec) and DHA (107.6±6.8 sec) rats held the pole firmly while the pole reached a 90° angle. MPTP (41.67±9.81 sec) animals tended to fall off in less than 40s. Nevertheless, the pole test latency was significantly increased in the MPTP+DHA (101.33±11.07 sec) group as compared to MPTP group. As presented in Fig. 1B, descent latency in the catalepsy test was significantly greater in MPTP (23.83±6.68 sec) rats than in control (1.33±0.33 sec) and DHA (1.83±0.31 sec) rats. Moreover, the cataleptic state was significantly decreased in MPTP+DHA (9.17±1.68 sec) group when compared to MPTP group.

Tyrosine hydroxylase immunohistochemistry

MPTP caused an obvious reduction in TH positive dopaminergic neuron viability as determined at day 7. The neuron numbers of the control group (Fig. 2A, E) were almost equivalent to the DHA group (Fig. 2D, H). Dopaminergic neuron numbers were clearly decreased in the MPTP group (Fig. 2B, F). DHA supplementation was effectively decreasing the dopaminergic neuron death in the MPTP+DHA group (Fig. 2C, G).

The compact, reticular and lateral parts of rat SN were easily distinguished by TH immunostaining. The immunoreactivity for TH was observed in neuron bodies and processes. No immunoreactivity was observed in glial cells and the endothelium. No differences in TH staining intensities were observed among groups. The staining intensities for TH immunoreactivity are presented in Table 1.

The immunostaining was quantitatively evaluated with the positive dopaminergic neuron staining with TH counted according to the experimental groups. The immunolabelling was significantly decreased in the MPTP group (152±2) that were found to be sparse and disorganized when compared to the control and DHA groups ($p<0.05$). In the MPTP+DHA group (309±2.4), the neuron processes were more organized and neuron number was significantly higher when compared to the MPTP group ($p<0.05$). In addition, TH immunopositive neuron number was higher in DHA groups (442±1.5) than the control groups (399±3). No staining was observed in the negative sections. Since the data from the TH positive cell counts were normally distributed, therefore one way ANOVA was used. Statistical calculations were performed using SigmaStat for Windows, version 3.0 (Jandel Scientific Corp. San Rafael, CA). Statistical significance was defined as $p<0.05$. The results are presented in Table 2.

Immunostaining of GDNF and NTN in SN

Immunohistochemical localizations of GDNF and NTN were prominent in the cytoplasm of dopaminer-

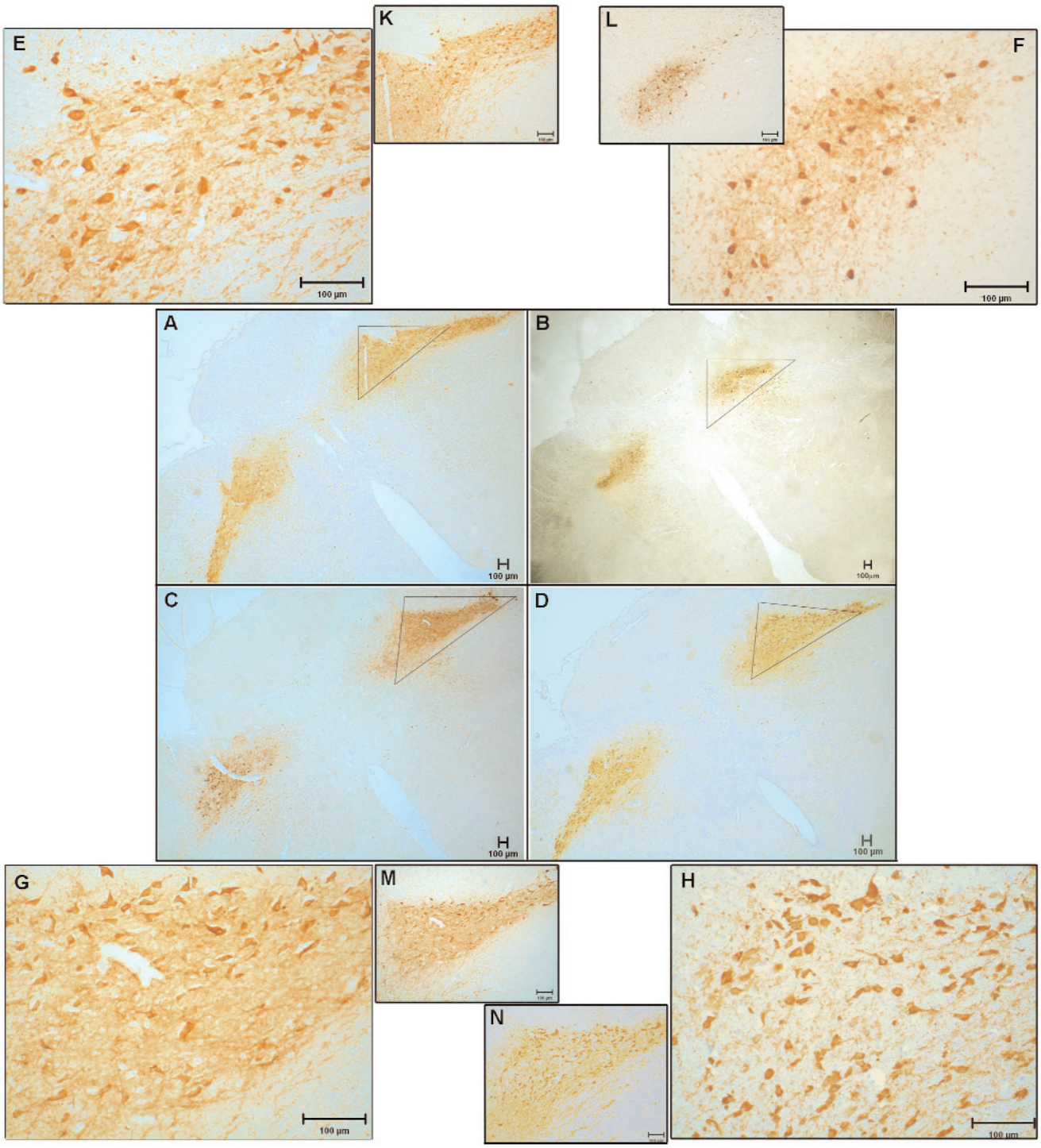

Fig. 2. Localization of tyrosine hydroxylase protein in dopaminergic neurons was presented. **A,E,K.** Control, **B,F,L.** MPTP, **C,G,M**. MPTP+DHA, **D,H,N**. DHA group. Magnification: A,B,C,D: ×2.5; E,F,G,H: ×40; K,L,M,N: ×20. Tyrosine hydroxylase immunoreactivity in the control group was almost the same as in MPTP+DHA group, which both were higher than that of the MPTP group. Scale bar represents 100 µm.

gic neurons with an increased expression in MPTP+DHA when compared to the MPTP groups.

Although, the dopaminergic neuron staining intensities were decreased in the MPTP group compared to the control (Fig. 3A *vs* 3B, Table 1), GDNF protein immunoreactivity was still present in MPTP treated neurons. Moreover, strong immunostaining for GDNF was observed in both the MPTP+DHA and DHA groups when compared to the MPTP group (Fig. 3C, D, 4C,D) NTN staining intensity was also decreased in

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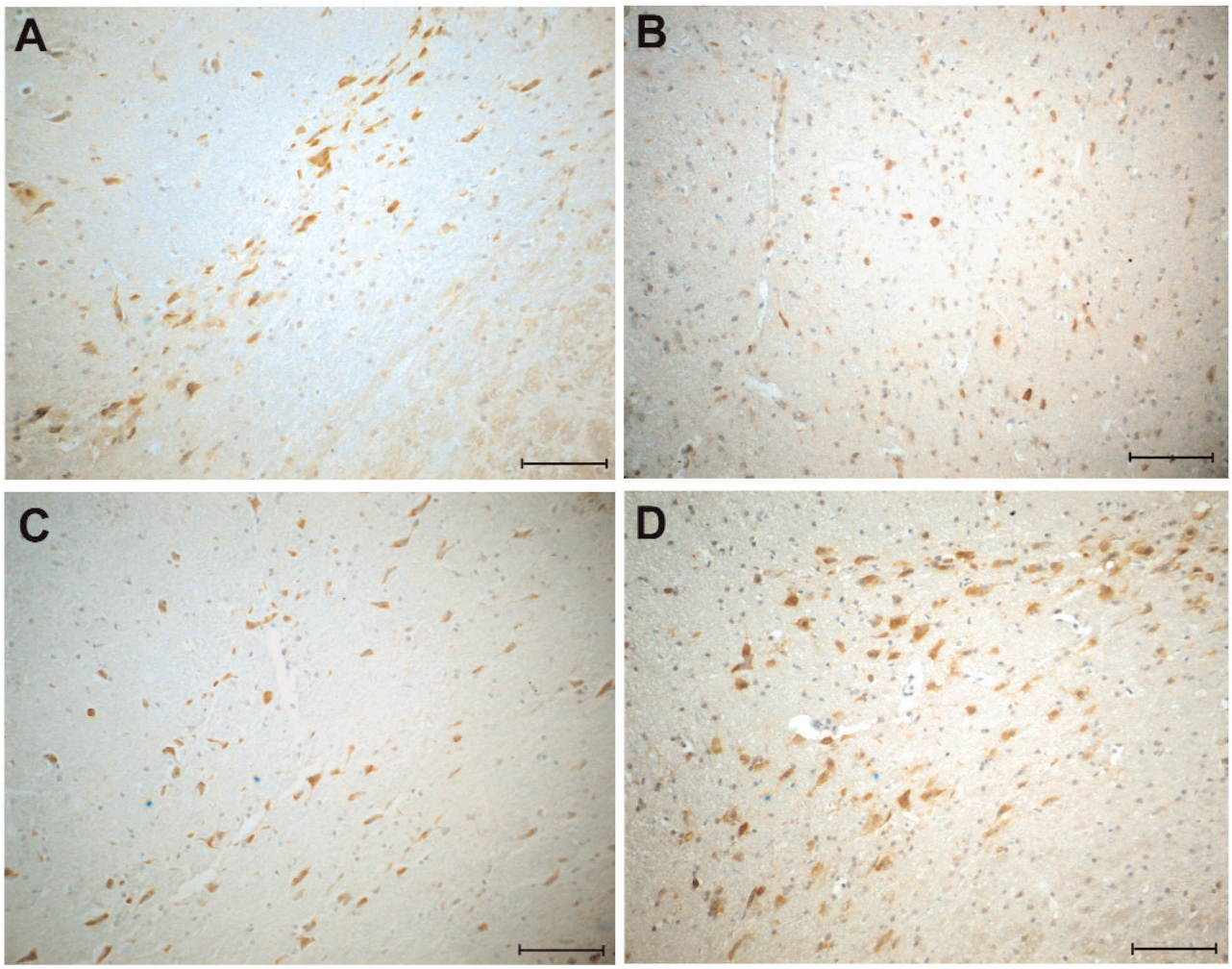

Fig. 3. Immunolocalization of GDNF in substantia nigra. **A.** Control, **B.** MPTP, **C.** MPTP+DHA, **D.** DHA group. Magnification: 20x. The immunoreactivity of GDNF was found to be higher in MPTP+DHA groups when compared to MPTP groups. In DHA group (D), the GDNF reactivity was higher than control group.

MPTP group compared to the MPTP+DHA, DHA and control groups (Fig. 5A-H and Table 1).

Discussion

The etiology of PD is probably a combination of environmental and genetic factors. MPTP represents the most important and most frequently used Parkinsonian toxin applied in animal models. The MPTP treatment affects mitochondria, either by inhibiting mitochondrial complex I or complex III [16] which leads to a significant reduction in the number of neurons in the *Substantia nigra pars compacta* (SNpc) [17]. In this study, we have also emphasized the reduction in TH-positive neuron numbers in the experimental PD model created by the toxic effects of MPTP in SN. TH is the rate-limiting enzyme in catecholamine synthesis. The most densely packed TH-positive cell area in the brain is the SNpc, which projects fibers to the striatum [18]. Since it has been shown that the cell-body rich SNpc primarily contains the soluble form of the TH enzyme, it is often used as the phenotypic marker for dopaminergic neuron numbers and can be measured both by biochemical and immunohistochemical methods to determine neuron loss [19].

Studies in animals clearly show that oral intake of DHA can alter brain DHA concentrations and thereby modify brain functions that is associated with memory loss and diminished cognitive function [20]. This provides us with an opportunity to use DHA as a nutraceutical or pharmaceutical tool in brain disorders such as PD [21]. Based on this hypothesis, we aimed to investigate the presence of DHA effects on dopaminergic neurons in SN after induced experimental Parkinson model, by using TH immunohistochemistry. Our TH immunohistochemistry results clearly indicated that chronic pre administration of DHA may partially restore dopaminergic neuron numbers in this experimental model of PD.

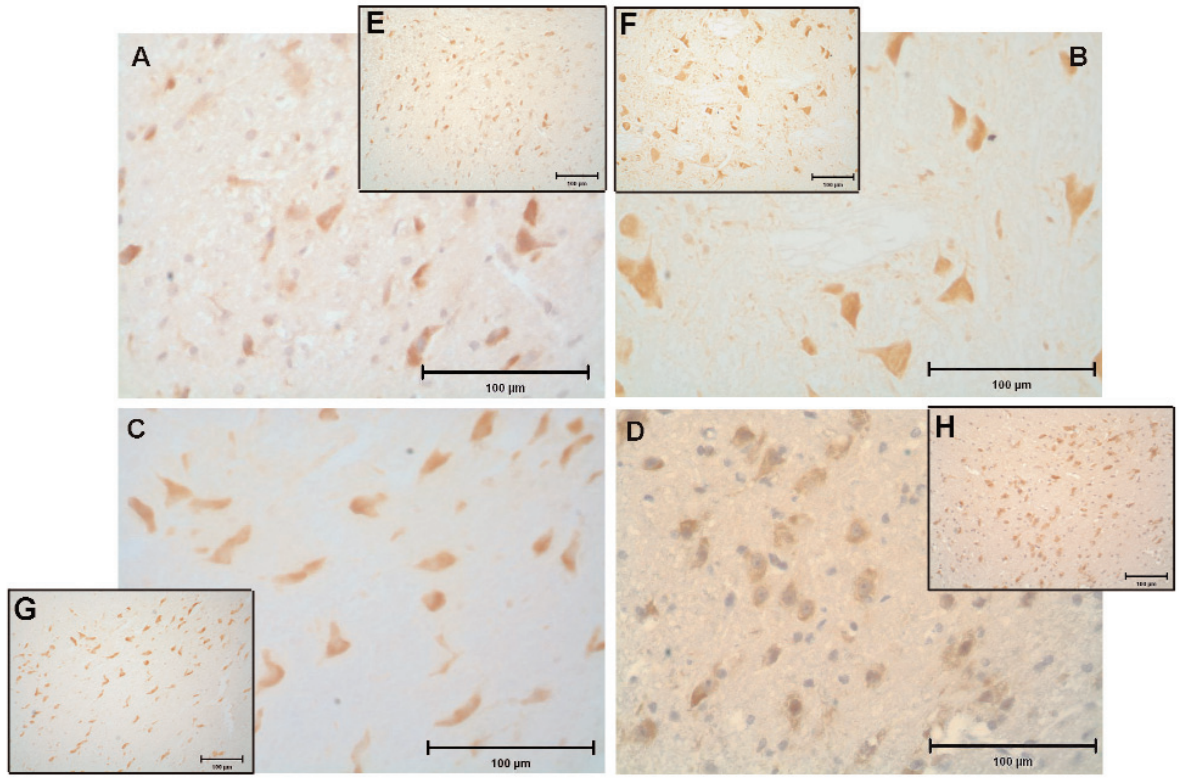

Fig. 4. Representative pictures were presented in a higher magnification (×40). **A-D.** GDNF immunolabelling in 4 groups. **A, E.** Control group. **B, F.** MPTP group. **C,G**. MPTP+DHA group. **D, H**. DHA group. Scale bar represents 100 µm.

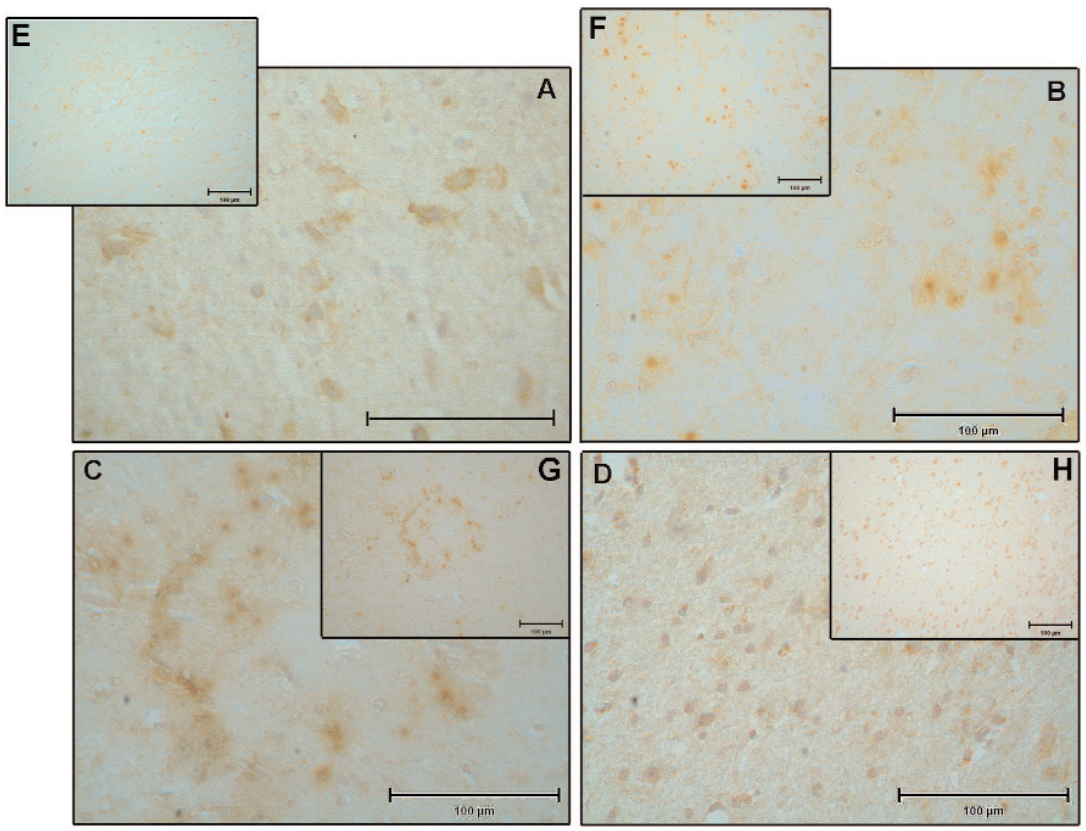

Fig. 5. Immunolocalization of NTN in substantia nigra. **A.** Control, **B.** MPTP, **C.** MPTP+DHA, **D.** DHA group. Magnification: ×20. NTN expression was cyctoplasmic and the expression was decreased in MPTP group compared to the MPTP+DHA group. Control and DHA groups had intense reactivity (A, D). E-H) A higher magnification of NTN immunolocalization was presented (×40). Scale bar represents 100 µm.

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We have also evaluated the motor activity in the experimental Parkinsonism model. Measurement of motor activity in experimental Parkinsonism models depends on the performance of animals in well defined tasks. The results of these test confirmed that the created model of PD was reliable. In our current study, MPTP-treated rats displayed typical behavioral characteristics of PD in the vertical pole and catalepsy test. The present findings are in agreement with results from previous studies which demostrated the impairment of motor activity in MPTP induced Parkinsonism model [22]. On the other hand DHA pre-administration reduced these symptoms in the MPTP group. This indicates a relationship between maintenance of TH positive cells and diminished Parkinsonism symptoms that were detected in the MPTP+DHA group. This DHA dose was selected from a previous study designed to represent human administration of high levels of DHA.

Neurotrophic factors regulate many critical aspects of the ontogeny of neurons, such as promoting survival, neurite branching and synaptogenesis [23]. One of those neurotrophic factors is GDNF which promotes the survival of the embryonic dopaminergic neurons of the midbrain during PD [24]. Therefore this trophic factor raised great expectations as a potential therapeutic agent for the treatment of neurodegenerative diseases [25]. In this study we have localized GDNF protein in the cytoplasm of dopaminergic neurons in SN. It is possible that the strong expression of GDNF in dopaminergic neurons might be related to its therapeutic effects in PD and might indicate that GDNF can successfully block the already initiated degenerative process in the SN. Our results showing the immunostaining of GDNF are consistent with the previous report by Zigmond *et al*. where they have shown the expression of GDNF in dopaminergic neurons suggesting a possible therapeutic effect. Furthermore, our results support Grondin and Gash (1998) who reported that GDNF rescues the dopaminergic neurons from the neurotoxin-induced death and stimulates functional recovery in an animal model of PD.

GDNF and the related factor NTN support several neuronal populations in the central nervous system, including midbrain dopamine neurons and motor neurons. In addition, these promote survival and regulate differentiation of many peripheral neurons [26]. GDNF and NTN can act as target derived trophic factors for dopaminergic neurons. These two factors show different and overlapping effects, because GDNF is a potent survival, neuritogenic and hypertrophic factor, while NTN only induces survival-promoting effect on nigral neurons [23]. Supporting these previous studies, we have observed that DHA treatment was effective in preventing the death of nigral dopaminergic neurons, likely to be shown with GDNF and NTN immunostaining intensities. Unfortunately, the exact mechanism of how DHA affects GDNF and NTN up-regulation is still unknown. Therefore, further functional studies are needed to clarify this issue.

In conclusion, DHA supplementation in MPTP-induced experimental Parkinsonism model significantly protects dopaminergic neurons against cell death by increasing the expressions of GDNF and NTN. Our data show that oral administration of DHA can prevent the loss in dopaminergic neurons. Moreover, the neuroprotective effects of GDNF and NTN combined with DHA will lead to better strategies for the treatment of neurodegenerative diseases. The present result is in agreement with results from previous studies which demonstrated a DHA enriched diet increased levels of brain derived neurotrophic factor (BDNF) [27]. The powerful neuroprotective and neurorestorative properties of GDNF and NTN suggest that tropic factors may play an important role in treating PD. We speculate that DHA administration prevents GDNF and NTN labelled dopaminergic neuron loss in an animal PD model.

Whereas the knowledge on the therapeutic potential of neurotrophic factors such as GDNF and NTN is still contradictory, it should be known that we are only at the beginning of the story.

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