Effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine on Phosphorylated Neurofilament in Brain Cortex of Rat Model for Parkinson's Disease

Samah M. Fathy[1,2*] and Ibrahim Y. Addelkader[3]

[1]*Zoology Department, Faculty of Science, Fayoum University, Fayoum, Egypt.*
[2]*Biology Department, Faculty of Science, Jazan University, Jazan, Kingdom of Saudi Arabia.*
[3]*Zoology Department, Faculty of Science, Cairo University, Giza, Egypt.*

Authors' contributions

This work was carried out in collaboration between both authors. Authors SMF and IYA designed the study and interpreted the data, author SMF wrote the protocol, anchored the field study, gathered the initial data and performed preliminary data analysis, managed the literature searches and produced the initial draft. Both authors read and approved the final manuscript

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting people over 60 years old. Although many studies on PD focused on basal ganglia, it has been reported that other brain regions might be involved. The current work investigated the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection on the brain cortex of PD rat model using Western blotting, immunohistochemistry and immunofluorescence techniques against phophorylated neurofilament antibody (pNF). A highly significant increase of pNF protein level in the treated rats was recorded using western blot as compared to the control rats. At the same time, Immunohistochemical and immunofluorescence analyses showed that the neuronal perikarya of the cerebral cortex of MPTP-treated rats were strongly immunopositive towards pNF. Meanwhile, axons and dendrites of the cerebral cortical neurons were selectively immunoreactive for pNF in

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Corresponding author: E-mail: samahfathy1972@gmail.com;

the control rats. This study suggested that MPTP injection increased the level of pNF protein and its perikaryal accumulation with axonal reduction in the cortex of PD rat model. These variations might have an important role in the understanding of the pathologic mechanism of PD.

1. INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affecting people over 60 years [1]. The main pathological symptom involves degeneration of the nigrostriatal dopaminergic neuronal pathway [2,3]. Recently, investigations of the pathologic mechanism of PD have mainly concentrated on the basal ganglia. However, latest studies have confirmed that pathological changes in PD are accompanied by changes of other brain regions such as the cerebral cortex. In addition, it was confirmed that alterations in the cerebral cortex is accompanied by functional motor disorders in PD [4].

It has been reported that neurotoxin1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) stimulates neuronal degeneration and mimics clinical and pathological features of PD [3]. The neurotoxic impact of MPTP is exerted via activation of monoamine oxidase enzyme resulting in the production of 1-methyl-4-phenyl-2,3-dihydropyrdinium ion (MPDP1) that is followed by 1-methyl-4-phenyl pyridinium (MPP1) metabolite release [3,5]. In addition, it is documented that MPTP suppresses mitochondrial respiration through complex I influence leading to loss of mitochondrial energy and subsequent cell death [6]. Moreover, MPTP induces the release of reactive oxygen species [7,8]. This induced MPTP neurotoxicity can be reversed via antioxidant enzymes activation [9].

NF proteins are member of cytoskeletal components that belong to an intermediate filament of a triplet protein; neurofilament light, medium and heavy chains respectively (NF-L, NF-M and NF-H) [10,11]. They are responsible for stiffness, tensile strength and possible management of intracellular transport to axons and dendrites [10].

Various neurodegenrative diseases are due to deficits in phosphatase and kinase activities and/or disruption of phosphorylation in different brain regions [12]. Hyperphosphorylated tau and pNF are obvious in the form of perikaryal inclusions in the CNS of people suffering from Alzheimer's disease (AD), PD and Amyotrophic lateral sclerosis (ALS) [12]. Moreover, neurofilament protein accumulation is considered as one of the first pathological variations in AD neurons [13,14].

It has been reported that MPTP neurotoxicity can provoke alterations in energy supply and cytoskeletal integrity of the neuronal cells causing disturbance of the normal cell functions [3]. However, to the best of our knowledge, the current study is a first-ever to investigate the effect of MPTP on pNF (H &M) level and distribution in the brain cortex of PD rat model.

2. MATERIALS AND METHODS

2.1 Experimental Design

This study was carried out in strict compliance with the Guidelines of Animals Health Research Institute, Egypt. The study protocol was approved by the Committee on the Ethics of Animals Health Research Institute, Egypt (Permit Number 362). Thirty six male Wistar rats (200-250 gm) were housed in a temperature controlled room under 12/12 hours light/dark cycle and had free access to food pellets and tap water ad libitum. The minimal number of animals needed to obtain statistical significance was used and all efforts were made to minimize animal suffering. On the day of experiment, rats were divided into two groups, one group received 4 i.p. injections of 15mg/kg each MPTP (sigma; ST. Louis, MO, USA) in saline at 2 hours intervals (total dose: 60 mg/kg) while the control rats were given equal dose of physiological saline only.

2.2 Immunoblot Analysis for pNF

Seven days later, rats were sacrificed by decapitation after narcotizing with aerosolized isoflurane and cerebral cortices were removed. Cerebral cortices (from six animals per group) were homogenized in the lysis buffer plus proteinase inhibitors. The homogenates were centrifuged to remove unbroken cells and debris. Supernatants were mixed with sample buffer containing 50 mM/l Tris-base (pH 6.8), 0.5% glycerol, 0.01% bromphenol blue, and 0.75%

sodium dodecyl sulfate (SDS) and heated at 95ºC for 5 min. Experiments were performed using the concentration of protein within the linear range for immunolabeling of target proteins. Equal amounts of protein from each group (15 ug/lane) were fractionated by 6% SDS–PAGE. The electrophoretically separated proteins were then transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat dry milk in TBST [50 mM Tris (pH 7.5), 150mM NaCl, and 0.1% Tween 20] buffer at pH 7.5 for 1 h at room temperature. The membranes were incubated overnight at 4ºC with a monoclonal primary antibody for pNF (MAB1592, Millipore), washed and incubated with the secondary antibody (sheep anti-mouse IgG) linked to alkaline phosphatase then followed by the color reaction.

2.3 Immunohistochemistry

Six animals from each group were sacrificed seven days postinjection, anesthetized and perfused transcardially at room temperature with 200 mL of phosphate-buffered saline (PBS; pH 7.4), followed by fixation with 200 mL of phosphate-buffered formalin, prepared by alkaline hydrolysis of paraformaldehyde (3.8% w/v; pH 7.2–7.4). The brains were then removed and postfixed in formalin for 2 h at 4ºC and rinsed overnight at 4ºC in PBS containing 20% (w/v) sucrose and 0.1% (w/v) sodium azide. Afterwards, they were embedded in OCT (Sakaura Finet). The cerebrum was divided coronally at the bregma, and 30-u*m* thick sections were cut on a cryostat microtome (Microm, Heidelberg, Germany) and further processed under free floating conditions. The sections were collected in phosphate buffer, washed in 0.6% hydrogen peroxide with 0.06% sodium azide, in PBS for 30 min to quench endogenous peroxidase activity, and rinsed in PBS (3x5 min) prior to immersion in 2% (w/v) bovine serum albumin (BSA; Vector Lab.). The braincortex sections were then incubated overnight at 4ºC with pNF (H & M) (MAB1592, Millipore, UK) monoclonal primary antibody at 1:50 dilution overnight at 4ºC while midbrain sections were incubated with anti tyrosine hydroxylase (anti-TH) monoclonal antibody (Millipore, UK) and subsequently with the biotinylated secondary antibody (Vector, 1:300) for 1 h at room temperature. Staining was visualized with diaminobenzidine after incubation with streptavidin - peroxidase. Negative immunohistochemical control sections were treated in the same way but in the absence of primary antibodies.

2.4 Immunofluorescence

Animals (six rats for each group) were anaesthetized with sodium pentobarbital and intracardially perfused with a saline solution followed by a 4% paraformaldehyde solution in phosphate buffer saline (PBS). Brains were post-fixed for 24 hours, transferred in 30% sucrose/PBS solution at 4ºC and then embedded in OCT (Sakaura Finet). Cryostat sections of thirty micrometers coronal sections were collected in 0.05% sodium azide/PBS in a culture well and stored at 4ºC until usage. Sections were washed in 1 X PBS for 10 min and then blocked in 0.1% Triton X-100, 15% normal goat serum in 1 X PBS for 1 h at RT. Sections were incubated with the monoclonal primary antibody in blocking solution overnight at 4ºC, washed in 1× PBS 3 × 10 min, and incubated in FITC-conjugated secondary antibodies for 2 h at RT. Sections were washed 3 × 10 min in 1× PBS and mounted in Vectashield (Vector Laboratories) that prevents rapid loss of fluorescence during microscopic examination. Omission of primary or secondary antibody from few sections had been applied and they were used as negative controls.

2.5 Statistical Methods

Results are presented as mean ± standard error of the mean. Statistical analysis was performed using an unpaired Student's t-test using a statistical package program (Sigma Plot version 11.0). Differences among groups were considered significant at $p < 0.05$.

3. RESULTS

Immunohistochemistry with an antibody selective for TH revealed significant reduction of the staining intensity in the substantia nigra (SN) of the MPTP-treated animals as compared with the control rats (Fig. 1).

Immunoblot analysis of the cortical homogenate using an antibody for the pNF (H & M) revealed a highly significant increase in the level of the protein (at 160 KDa, **$P < 0.01$ and 200 KDa, ***$P < 0.001$) in cortex of MPTP-treated rats compared with that in cortex of the control rats (Fig. 2). Meanwhile, Immunohistochemical analysis of the control cerebral cortex showed that axons and dendrites of the neurons in layers II–VI were selectively immunoreactive for pNF,

while the perikarya lacked immunoreactivity (Fig. 3A) except for few interneurons. Following MPTP injection, pNF immunoreactivity was accumulated in the neuronal perikarya of layers II–IV in the temporal cerebral cortex (Figs. 3B and 3C), as well as in other cortical regions. Immunoflurescence technique revealed axonal loss in the cortical region of the MPTP-treated rats as compared with the control ones (Fig. 4).
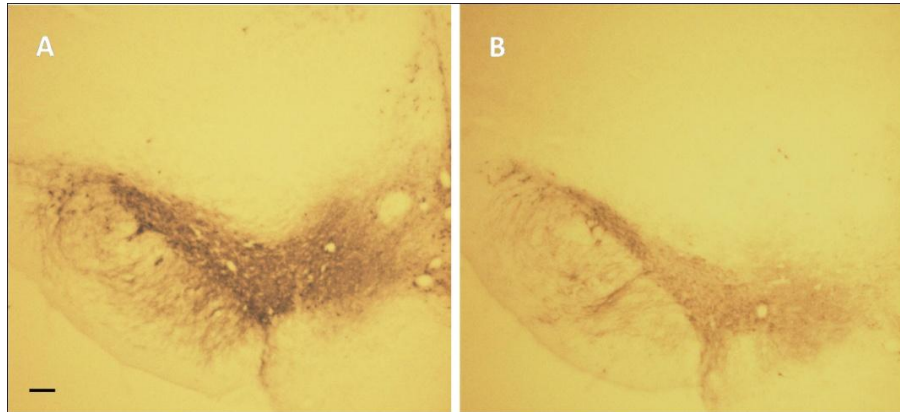

Fig. 1. TH immunoreactivity in the substantia nigra (SN). (A) Saline treated, (B) MPTP treated rats. Scale bar= 100 *µ*m
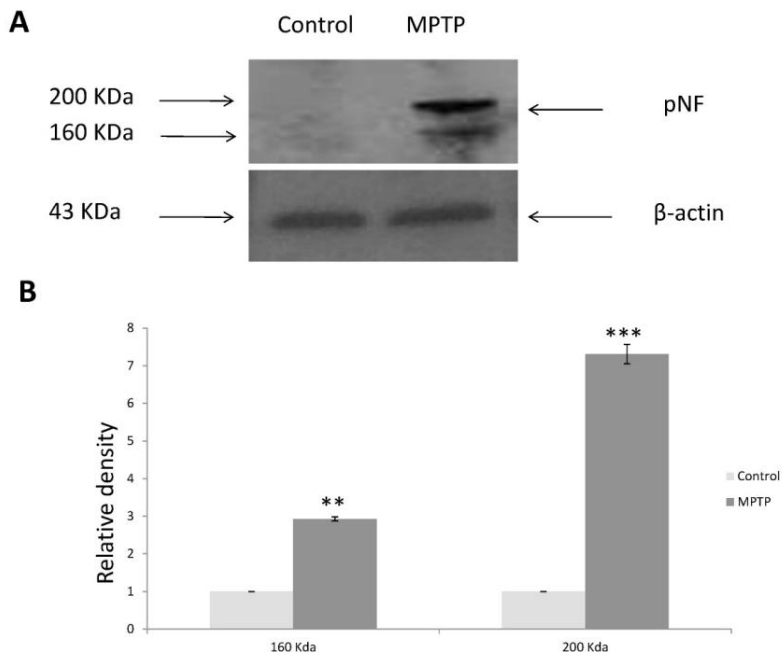

Fig. 2. MPTP increased pNF (H & M) level. (A) Representative Western blots for pNF (H & M) protein expression in brain cortex (B) Quantification of the Western blot for pNF (H & M). The densitometry of pNF (H & M) bands was first normalized to the loading control β-actin. The relative density of the expressions of pNF (H & M) in the MPTP-treated rats relative to that in the control rats was then calculated. The scanned image of Western blot was analyzed with the software Image J. Data were presented as mean ± SEM. **P< 0.01, ***P< 0.001, MPTP-treated group versus control group
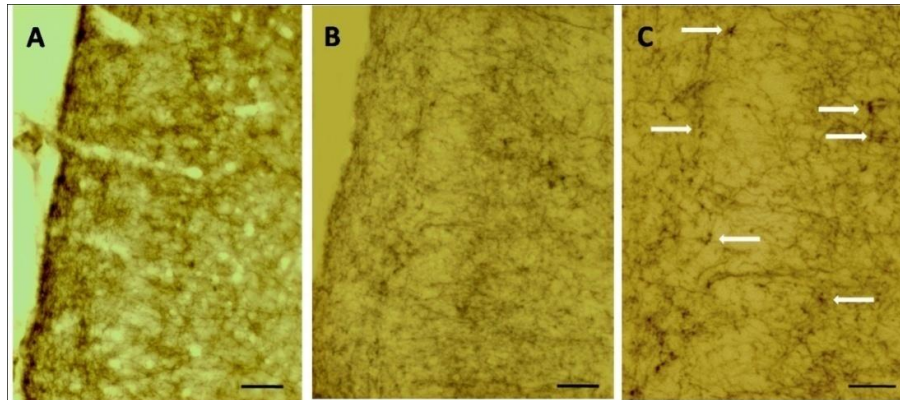
Fig. 3. Phosphorylated-NF immunoreactivity in the temporal cerebral cortex. Control rats (A), the immunoreactivity is restricted to neuronal processes. MPTP treated rats (B), accumulation is immunoreactivity in neuronal perikarya. Higher magnification revealed that the neuronal perikarya were strongly stained (arrows) (C). Bar = 70 μm (A, B), 40 μm (C)
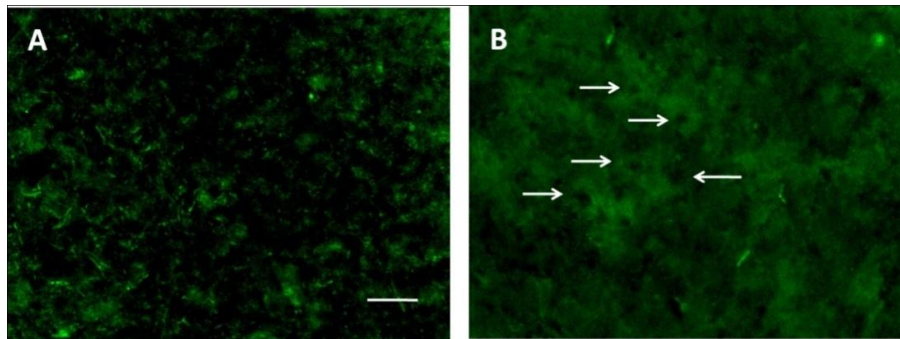

Fig. 4. Immunofluorescence analysis towards pNF antibody. Control animals (A) shows high immunoreactivity at the neuronal processes. MPTP treated rats (B) with perikaryal accumulation (arrows) and reduced processes immunoreactivity. Bar = 40 μm

4. DISCUSSION

It has been reported that NF abnormalities are associated with cellular disorder in many neurodegenerative diseases including PD [10,15]. Formation of Lewy bodies (LBs) and alpha-synuclein aggregation are considered as major neuropathological features of PD [16]. The three NF subunits represent main constituents of LBs [10,15]. Ubiquitin and proteosome subunits are also demonstrated [17]. Moreover, the biochemical investigations and electron microscope studies have confirmed the formation of a non-membrane-bounded compacted tangle in the perikarya of the affected neurons due to the abnormally pNF-H [10]. Consequently, the removal of the protein aggregates could be a vital factor in the disease control. This will be achieved via understanding the filament formation and abnormalities in response to different insults to reach to an effective therapeutics.

Phosphorylation of NF protein is controlled within neurons. Various studies showed that degree of NF phosphorylation is associated with filament translocation, formation, function as well as provocation of some neurodegenerative disorders [10]. Little studies dealt with alteration of pNF after MPTP injection especially in rat model for PD. In the current study, MPTP injection induced PD pathology in rats as that was confirmed by the reduction of TH immunoreactivity in the SN region. The obtained results are in harmony with previous studies which showed similar impacts for MPTP on the SN in primates and mice [18-20].

Furthermore, the present work recorded substantial elevation of pNF protein expression in the cortex as well as abnormal accumulation at

neuronal perikarya following MPTP treatment. Duong et al. [21] demonstrated the formation of Intraneuronal tangle with neurofilamentous accumulation in the epileptic cortical dysplasia and AD individuals.

Moreover, these alterations were also observed in different brain regions of other neurodegenerative diseases such as ALS, diabetic neuropathy and giant axonal neuropathy [22-27]. Such these changes might be directly or indirectly linked with the disease pathology. These alterations also might be due to the production of the toxic metabolite MPP+, whose release is controlled by MAO enzyme [5]. Luigi et al. [3] showed that subtoxic dose of MPTP reduced axon outgrowth and led to hyperphosphorylation of NF-H in differentiating mouse N2a neuroblastoma cells.

Additionally, the irregular distribution of pNF in cortical neurons triggered by MPTP might be followed by neuronal loss due to abnormal trafficking of the cytoskeletal protein. It was shown that higher doses (>100 um) of MPTP induced neuronal death in differentiating mouse N2a neuroblastoma cells [3]. Mhatre et al. [11] demonstrated similar changes after thrombin exposure. It has been reported that changes in the phosphorylation state of NF lead to axonal destruction [28].

Moreover, these alterations might be attributed to various signaling cascades. In this context, Giassson and Mushnyski [29] suggested that alterations in NF phosphorylation following neurotoxicity might be due to the activation of c-Jun N-terminal kinase (JNK) signaling pathway. The results of the current experimental work might be encouraging to examine the impact of MPTP injection on pNF protein at other brain regions in PD rats.

5. CONCLUSION

In conclusion, MPTP treatment induces PD pathology in rat and affects the phosphorylation state of cytoskeletal proteins at brain cortex. It causes aberrant accumulation of pNF in the neuronal perikarya that might be followed by neuronal loss. These changes might lead to more understanding of the pathologic mechanism of PD.

COMPETING INTERESTS

All authors declare that there is no conflict of interest as regards; financial or personal relationships for other people or organizations.

Nobody except the authors is involved in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication and there are no funding resources.

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