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Minocycline, levodopa and MnTMPyP induced changes in the mitochondrial proteome profile of MPTP and maneb and paraquat mice models of Parkinson's disease

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ABSTRACT

Mitochondrial dysfunction is the foremost perpetrator of the nigrostriatal dopaminergic neurodegeneration leading to Parkinson's disease (PD). However, the roles played by majority of the mitochondrial proteins in PD pathogenesis have not yet been deciphered. The present study investigated the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and combined maneb and paraquat on the mitochondrial proteome of the nigrostriatal tissues in the presence or absence of minocycline, levodopa and manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP). The differentially expressed proteins were identified and proteome profiles were correlated with the pathological and biochemical anomalies induced by MPTP and maneb and paraquat. MPTP altered the expression of twelve while combined maneb and paraquat altered the expression of fourteen proteins. Minocycline, levodopa and MnTMPyP, respectively, restored the expression of three, seven and eight proteins in MPTP and seven, eight and eight proteins in maneb- and paraquat-treated groups. Although levodopa and MnTMPyP rescued from MPTP- and maneb- and paraquat-mediated increase in the microglial activation and decrease in manganese-superoxide dismutase expression and complex I activity, dopamine content and number of dopaminergic neurons, minocycline defended mainly against maneb- and paraquat-mediated alterations. The results demonstrate that MPTP and combined maneb and paraquat induce mitochondrial dysfunction and microglial activation and alter the expression of a bunch of mitochondrial proteins leading to the nigrostriatal dopaminergic neurodegeneration and minocycline, levodopa or MnTMPyP variably offset scores of such changes.

1. Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the selective degeneration of dopaminergic neurons of the nigrostriatal pathway. Resting tremor and impaired movement and coordination are reported to be the major symptomatic features of the disease [1,2]. Loss of dopaminergic neurons depletes dopamine content in the dorsal striatum [1]. Anatomically, the disease is characterized by the formation of intra-cytoplasmic protein aggregates called Lewy bodies, in the adjacent neurons [1]. While PD is mainly an aging related disease, inputs of the genetic factors and environmental exposure to pesticides and heavy metals have also been well documented [2]. Several animal models have been developed to study the cellular and molecular pathogenesis and to ascertain the effective therapy to encounter PD [2]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) not only reproduces some of the basic PD features in primates and rodents but is also regarded as a well established model [1]. Similarly, two commonly used pesticides namely manganese ethylene-bis-dithiocarbamate (maneb) and N,N′-dimethyl-4,4′-bipyridinium dichloride (paraquat)

Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Maneb, manganese ethylene-bis-dithiocarbamate; Paraquat, N,N′-dimethyl-4,4′-bipyridinium dichloride; MnTMPyP, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin; Mn-SOD, manganese superoxide dismutase; AOP1, antioxidant-like protein 1; Prx3, peroxiredoxin 3; IDH3α, isocitrate dehydrogenase 3 (NAD^+) α; VDAC, voltage dependent anion channel; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium salt; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; BSA, bovine serum albumin; DHBA, 3,4-dihydroxybenzylamine hydrobromide; $MgCl_2$, magnesium chloride; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; IPG, Immobiline pH gradient; NaCN, sodium cyanide; KH_2PO_4, potassium dihydrogen orthophosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide disodium salt; TH, tyrosine hydroxylase; PVDF, Polyvinylidene difluoride; CBB, coomassie brilliant blue; SDH, succinate dehydrogenase; LDH, lactate dehydrogenase; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF/TOF, matrix assisted laser desorption/ionization-time of flight/time of flight; SEM, standard error of means; DLST, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex; COX 5a, cytochrome c oxidase subunit 5a; DRP-2, dihydropyrimidinase-related protein-2; Prx2, peroxiredoxin 2; PDH E1α, pyruvate dehydrogenase E1 component subunit α; PEBP1, phosphatidylethanolamine-binding protein 1; UCH-L1, ubiquitin carboxyl-terminal hydrolase isozyme-L1

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in combination also mimic several cardinal features of PD in mice [2]. The combined maneb and paraquat model is environmentally relevant and exhibits the slow and progressive nigrostriatal dopaminergic neurodegeneration similar to sporadic PD [2].

Due to multi-factorial and sporadic nature of PD, all animal models developed so far have some or the other limitations [2]. While transgenic models elucidate the roles played by the selected genes, pathological aberrations in sporadic PD are contributed by multiple genes rather than a few selected genes [3,4]. MPTP and combined maneb and paraquat models were selected over the transgenic models, as environmental toxins are implicated in PD pathogenesis [1,2]. While rotenone model is environmentally relevant and is better than MPTP or combined maneb and paraquat in a few aspects, such as distinct Lewy body formation, it non-specifically affects the brain. MPTP and combined maneb and paraquat models were preferred over the rotenone model since MPTP and paraquat are structurally alike, their preferential target is the nigrostriatal dopaminergic neurons and they lead to the mitochondrial dysfunction and subsequent free radical generation almost in the similar fashion [1,2].

The mitochondrion acts as an epicenter of PD pathogenesis since its impaired function is associated with sporadic PD and a few toxicant-induced PD models [1]. MPTP and combined maneb and paraquat inhibit the mitochondrial complex I and/or III [2,5,6] and induce oxidative stress, neuroinflammation and microglial activation, which subsequently lead to the nigrostriatal dopaminergic neurodegeneration [2,7]. Albeit MPTP and combined maneb and paraquat inhibit the mitochondrial complex I, underlying mechanisms have been found to be unrelated. MPTP inhibits ATP biosynthesis owing to the inhibition of electron transfer from iron–sulfur cluster of complex I to ubiquinone [8] while paraquat generates free radicals mainly through redox cycling by withdrawing the electron from the mitochondrial complex I enzyme [6]. Such differences raise the possibility that diverse steps could be involved after complex I inhibition in both the models.

Proteomic approaches have been used to identify MPTP-induced changes in the expression of multiple proteins associated with defective energy metabolism, ubiquitin proteasome system, apoptosis and mitochondrial function [2]. A few proteins, which decide the fate of the genetic forms of PD, are localized in or interact with the mitochondria and mitochondrial dysfunction could affect neuronal survival [9]. MPTP and maneb and paraquat have been shown to affect the mitochondrial protein complexes, their effects on the proteins of the mitochondrion and the roles of such proteins in the mechanisms of neurodegeneration have not been fully characterized. Mitochondrial proteomics could be used as a precise tool to explicate the inputs of the novel mitochondrial proteins in MPTP- or maneb- and paraquat-induced PD.

Minocycline is a clinically available antibiotic and is an anti-inflammatory molecule. Its neuroprotective efficacy is reported against many neurological disorders employing animal models [10]. Minocycline is also used in animal models of Parkinsonism and found to inhibit microglial activation and neuroinflammation, two key events involved in PD pathogenesis [11,12]. Therefore, the present study investigated the effect of minocycline against toxin-induced rodent models of PD. Similarly, levodopa, a dopamine precursor (in combination with 3,4-dihydroxyphenylalanine decarboxylase inhibitor-carbidopa), is extensively used to ameliorate motor dysfunction and other deleterious effects of dopamine depletion against chemically-induced Parkinsonism [13–15]. While levodopa is used to improve symptomatic features, conflicting reports are available in literature and a few studies failed to detect protective or toxic effect [13]. Toxic potential of levodopa is described in culture cells but in vivo experimentations failed to unequivocally demonstrate whether levodopa accelerates degeneration of dopaminergic neurons of the substantia nigra and causes permanent impairment of their function or not [13]. Effect of levodopa largely depends on the route of its administration, dose and time of exposure [16].

Role of oxidative stress in pesticides-induced Parkinsonism and altered level of superoxide dismutase (SOD) in PD patients are widely reported [6,17,18]. Use of a levodopa or SOD/catalase mimetic, such as MnTMPyP, could rescue a compromised antioxidant defense system. Moreover, superoxide ion is produced as a result of electron transfer reactions occurring within the mitochondria, assessment of the effect of MnTMPyP on the mitochondrial proteome could highlight the link between oxidative stress and mitochondrial dysfunction. Deficiency of manganese-superoxide dismutase (Mn-SOD) or its altered expression is also associated with oxidative stress caused by paraquat or MPTP [6,17]. Metalloporphyrins, synthetic SOD/catalase mimetics, protect against MPTP- and paraquat-induced toxic effects in rodents [19,20]. Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP), a metalloporphyrin, inhibits lipopolysaccharide-induced free radical generation and dopaminergic neurodegeneration [21]. Chemical entities, which restore dopamine content, attenuate oxidative stress and inhibit microglial activation, could resist the mitochondrial dysfunction and subsequent events leading to neurodegeneration [17,22,23]. Albeit several studies are performed to measure the neuroprotective efficacies of minocycline, levodopa and MnTMPyP against PD [12,14,21], studies relating with their effects on the mitochondrial proteome have been limited. A few studies based on the mitochondrial proteomics are conducted using MPTP model [24–26] but not even a single study describing the mitochondrial proteome profile of combined maneb and paraquat model is reported to date with the best of our knowledge. Comparative mitochondrial proteome patterns of MPTP and maneb and paraquat at the doses, which induce PD phenotype, could offer clues to understand the similarities and discrepancies between the two. Moreover, changes in the mitochondrial proteome profiles of MPTP and combined maneb and paraquat in the presence or absence of levodopa, minocycline and MnTMPyP could help in identifying their variable effects and mode of actions and elucidating the disparities, if any.

2. Experimental procedures

2.1. Chemicals

Acetonitrile, acrylamide, ammonium bicarbonate, ammonium persulphate, anti-antioxidant-like protein 1 (AOP1)/peroxiredoxin (Prx) 3, anti-isocitrate dehydrogenase 3 (NAD+) α (IDH3α) and anti-voltage dependent anion channel (VDAC) primary antibodies, anti-mouse/rabbit biotin conjugated secondary antibody, antimycin, MPTP, alkaline phosphatase chromogen containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) liquid substrate, bromophenol blue, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 3,3′-diaminobenzidine liquid enhanced system, 3,4-dihydroxybenzylamine hydrobromide (DHBA), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), fatty acid free bovine serum albumin (BSA), Folin Ciocalteu's reagent, 3-hydroxytyramine hydrochloride, magnesium chloride ($MgCl_2$), mannitol, maneb, N,N′-methylene bisacrylamide, NBT salt, nonidet P-40, paraformaldehyde, paraquat, protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), rotenone, sodium cyanide (NaCN), sodium deoxycholate, sodium dodecyl sulfate (SDS), sodium fluoride, sodium orthovanadate, sodium pyruvate, sodium succinate, tris-base, N,N,N′,N′-tetramethylethylenediamine, trifluoroacetic acid (TFA), tween-20, triton X-100, ubiquinone and urea were procured from Sigma-Aldrich, St. Louis, MO, USA. Immobiline pH gradient (IPG) strips, IPG buffers and dry strip cover fluid were obtained from GE Healthcare, Chalfont, St. Giles, UK. Copper (II) sulfate 5-hydrate, formaldehyde, glycerol, methanol, MnTMPyP, potassium chloride, potassium dihydrogen orthophosphate (KH_2PO_4), potassium sodium tartrate, silver nitrate, sodium carbonate and sodium thiosulphate were procured from Merck Biosciences, Darmstadt, Germany. Acetic acid, agarose, cytochrome c, dibutyl phthalate xylene, disodium hydrogen

phosphate, ethanol, ethylacetate, heptane sulphonic acid, isobutanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), reduced nicotinamide adenine dinucleotide disodium salt (NADH), sucrose, sodium chloride, sodium dihydrogen orthophosphate, thiourea and trichloroacetic acid were procured from Sisco Research Laboratories Private Limited, Mumbai, India. Horseradish peroxidase conjugated streptavidin and normal goat serum were obtained from Bangalore Genei, Bangalore, India. Acetone, hydrogen peroxide, orthophosphoric acid, perchloric acid and xylene were obtained from Thermo Fisher Scientific Pvt. Ltd, Rockford, IL, USA. Anti-Mn-SOD, anti-stathmin, anti-tyrosine hydroxylase (TH), anti-integrin-αM/OX-42 primary antibodies and anti-rabbit and anti-mouse alkaline phosphatase conjugated-secondary antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Trypsin (sequencing grade) was obtained from Promega, USA. Polyvinylidene difluoride (PVDF) membrane was procured from Millipore, Billerica, MA, USA and frozen section medium Neg-50 from Richard Allen Scientific, Kalamazoo, MI, USA. Coomassie brilliant blue (CBB) R-250 was purchased from VWR International Limited, UK. Minocycline and levodopa (in combination with carbidopa) were of pharmaceutical grade and purchased locally.

2.2. Animal treatment, isolation of brain and dissection of the nigrostriatal tissues

Male Swiss albino mice were obtained from animal colony of the CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, India. Mice were kept under standard conditions, as described elsewhere [27]. Animals were treated intraperitoneally with MPTP (30 mg/kg), once a day, for 15 days or with maneb (30 mg/kg) and paraquat (10 mg/kg), twice a week, for 9 weeks along with respective controls [28,29]. The treatment schedules of MPTP and combined maneb and paraquat were selected in order to achieve the comparable level of dopamine depletion and the selected doses are also in accordance with the previously published studies [27,28]. Subsets of animals were also treated intraperitoneally with levodopa (carbidopa combined; total 7 mg/kg) or minocycline (30 mg/kg) or MnTMPyP (5 mg/kg) [11,30,31], once a day, for 15 days in case of MPTP and for 9 weeks in case of maneb- and paraquat-treated groups along with respective controls. Levodopa, minocycline or MnTMPyP was administered 2 h before the administration time of the toxin. Controls were injected with an equal volume of vehicles using the similar treatment paradigms. Animals were killed via cervical dislocation and brain was dissected out and kept at −80 °C till further use. The substantia nigra and dorsal striatum were dissected out from the brain. Enzyme activities and dopamine content were measured on the same day; however, remaining experiments were performed a few days after the day of animal killing. The study (a part of BSC0115) was approved by the institutional ethics committee for the use of laboratory animals.

2.3. Immunostaining of dopaminergic neurons and activated microglia

TH and integrin-αM specific antibodies were used to stain dopaminergic neurons and microglial cells, respectively, of the substantia nigra in the coronal sections of mouse brain. TH- and integrin-αM-immunoreactivities were performed, as described elsewhere [27]. Sections were viewed under the microscope (Leica Microscope DM6000 B, Germany), images were captured and the cells were counted using Leica QWin image analysis software (Leica Microsystems, Heerbrugg, Switzerland), as described previously [11,27]. In summary, the slides were coded by a person and counting was done in an unbiased way by another person in the area recognized by the anatomical landmarks. The first section from each brain was selected from a fixed distance. Thereafter, every second section was selected and TH-positive cells/microglial cells were counted bilaterally using two sampling areas (100 μm × 100 μm) on one side for each tracing. Counting was performed in three sections per animal using 3 animals per group per set of experiment and a minimum of 3 independent sets of experiments were performed [11,27].

2.4. Measurement of dopamine

Dopamine was measured (n = 3 independent sets comprised of the striatal tissue of 2 animals per set) in filtrate of the supernatant, which was collected after centrifugation of the striatal tissue homogenate (10% w/v), employing high performance liquid chromatography coupled with reverse phase C-18 column and electrochemical detector (Waters, Milford, MA, USA) [27]. DHBA was used as the internal standard and 3-hydroxytyramine hydrochloride as the external standard.

2.5. Isolation of mitochondrial fraction

Mitochondrial fraction was isolated from the nigrostriatal tissues (dorsal striatum and substantia nigra) by differential centrifugation [32]. The tissues were homogenized in the mitochondria isolation buffer (pH 7.2) containing HEPES (20 mM), sucrose (75 mM), mannitol (215 mM), EGTA (1 mM), EDTA (1 mM), PMSF (1 mM) and protease inhibitor cocktail. Homogenate was centrifuged at 1000 ×*g* for 5 min at 4 °C and the supernatant was collected. The pellet was re-suspended in the same buffer, centrifuged and the supernatant was taken. The supernatants were pooled and centrifuged at high speed (13,000 ×*g*) for 10 min at 4 °C. The pellet was washed twice with the same buffer and suspended in a minimum volume of the mitochondria isolation buffer. The suspension was challenged with repeated freezing and thawing followed by sonication to release the mitochondrial proteins.

2.6. Measurement of protein content

Protein content was measured by Lowry's method using the standard curve of BSA [33].

2.7. Succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) assays

Isolated mitochondrial or cytoplasmic fraction (n = 3 independent sets and the nigrostriatal tissues of 3 animals were pooled to make one set) was suspended in SDH buffer (pH 7.4) containing sodium phosphate (0.25 M), fatty acid free BSA (5 mg/ml) and distilled water [24]. Iodonitrotetrazolium (1% w/v) was added to the reaction mixture and vortexed. Sodium succinate (100 mM) was added, mixed and the reaction mixture was incubated at 37 °C for 90 min. Trichloroacetic acid (10% w/v) was added to the reaction mixture to stop the reaction. Ethylacetate was added to obtain an organic phase, which was used to read absorbance at 490 nm. For LDH assay, isolated mitochondrial or cytoplasmic fraction was mixed with sodium pyruvate (27 mM) and phosphate buffered saline. NADH (4 mM) was added to initiate the reaction and change in absorbance was recorded at 340 nm for 3 min. The SDH or LDH specific activity was calculated in terms of nmol/min/mg protein. LDH or SDH activity was also assayed in the total homogenate. The value of the total homogenate was considered as 100% and the specific activity of LDH or SDH was calculated accordingly.

2.8. Complex I and complex III activities

Complex I and III activities were performed from the isolated pure mitochondrial fractions (n = 3 independent sets and the nigrostriatal tissues of 3 animals were pooled to make one set). For complex I activity, isolated mitochondria and ubiquinone 1 (0.05 mM) were added to the assay buffer (pH 7.4) containing potassium phosphate (35 mM), NaCN (2.65 mM), $MgCl_2$ (5 mM), EDTA (1 mM), fatty acid free BSA

(1 mg/ml) and antimycin (2 μg/ml). The reaction mixture was incubated for 2 min and NADH (5 mM) was added to initiate the reaction. The rate of decrease of absorbance at 340 nm was monitored (SpectraMax M5 spectrophotometer, Molecular Devices, USA) for 3 min at the interval of 15 s. The enzyme activity was calculated as nmol of NADH oxidized/min/mg protein and expressed as % of control [34].

Complex III activity was measured employing a method described elsewhere [35] with minor modifications. Isolated mitochondria were added to the reaction mixture containing potassium phosphate (50 mM; pH 7.4), EDTA (10 mM), $MgCl_2$ (5 mM), NaCN (2 mM), rotenone (1 μM) and cytochrome c (1.25 mg/ml). The reaction was initiated by the addition of ubiquinol (100 μM). The absorbance was recorded (SpectraMax M5 spectrophotometer, Molecular Devices, USA) at 550 nm for 5 min. The enzyme activity was calculated as nmol of cytochrome c reduced/min/mg protein and expressed as % of control.

2.9. Proteome profiling

Mitochondrial proteins (n = 3 independent sets and the nigrostriatal tissues of 3 animals were pooled to make one set) were separated employing two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and differentially expressed proteins were identified by matrix assisted laser desorption/ionization-time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry.

2.9.1. 2-D PAGE

Mitochondrial proteins (~400 μg) were mixed with chilled acetone (1:10 ratio) and the mixture was kept at −20 °C for 2 h to precipitate the proteins. Mitochondrial proteins were concentrated by centrifuging the same at 15,000 ×*g* for 20 min at 4 °C and dissolving the resultant pellet in a minimum volume of 2-D PAGE lysis buffer (4% CHAPS, 1 mM EDTA, 7 M urea, 2 M thiourea, 20 mM tris, 10 mM DTT, 1 mM PMSF and protease inhibitor cocktail). The mixed content was kept at room temperature for 1 h to solubilize the proteins. IPG strips were rehydrated in the rehydration buffer (2% IPG buffer, 2% CHAPS, 0.002% bromophenol blue, 8 M urea and 18 mM DTT) containing mitochondrial proteins for 8 h and the isoelectric focusing was performed up to 14,000 V h. IPG strips were equilibrated [29] in the equilibration buffer [50 mM Tris–HCl (pH 8.8), 30% glycerol, 6 M urea, 2% SDS, 0.002% bromophenol blue and 65 mM DTT] for 20 min and the second dimension electrophoresis was carried out in 12.5% SDS polyacrylamide gels. CBB R-250 or silver staining was done to visualize the protein spots in the gels. The analysis of resultant spots was done using image master 2-D platinum software version 7.0 [11,29].

2.9.2. MALDI-TOF/TOF analysis

The differentially expressed protein spots were excised from the CBB R-250 stained gels, chopped into tiny pieces and destained until blue color disappeared. Gel pieces were rinsed with ammonium bicarbonate (25 mM) and dehydrated thrice with the equal volume of acetonitrile and ammonium bicarbonate. Gel pieces were dried and trypsin (2 μg/μl) and ammonium bicarbonate (25 mM) were added and incubated overnight at 37 °C to digest the proteins. The mixed content was centrifuged and the supernatant was taken. The pellet was suspended in 60% acetonitrile containing 1% TFA; incubated for 15 min and centrifuged to obtain the supernatant. Supernatants were pooled and speed vac concentrated. Concentrated trypsinized peptides were dissolved and co-crystallized with the matrix, i.e., α-cyano-4-hydroxy cinnamic acid. MALDI-TOF/TOF was performed in the reflectron mode (model 4800, ABsciex, USA) and the protein identity was established, as described elsewhere [29].

2.10. Western blotting of IDH3α, Prx3, stathmin and Mn-SOD

Western blotting was performed according to the method described elsewhere [27]. Isolated mitochondria (n = 3 independent sets and the nigrostriatal tissues of 3 animals were pooled to make one set) were suspended in radioimmunoprecipitation assay buffer [pH 7.6; Tris–HCl (50 mM), sodium chloride (150 mM), EDTA (2 mM), EGTA (2 mM), nonidet P-40 (1% v/v); triton X-100 (1% v/v), sodium fluoride (10 mM), sodium orthovanadate (2 mM), sodium deoxycholate (1% w/v), SDS (1% w/v) and PMSF (1 mM)]. Suspended sample was sonicated and centrifuged at 100,000 ×*g* for 1 h to isolate the membrane bound proteins as well as matrix associated proteins. The protein content was measured as mentioned in the preceding section and ~50 μg protein was subjected to SDS-PAGE. The resultant gel was electro-blotted onto PVDF membrane. Non-fat dry milk (5% w/v) and tween-20 (0.1% v/v) in tris buffered saline were used to block the non-specific binding. After successful electro-transfer [as detected by Ponceau S (1.1% w/v in 1% v/v acetic acid) staining], the membrane was incubated with respective primary antibody (anti-VDAC, dilution: 1:700; anti-IDH3α, dilution: 1:2000 or anti-AOP1, dilution: 1:5000 or anti-stathmin, dilution: 1:500, anti-Mn-SOD, dilution: 1:2000) for 3–4 h at room temperature. The membrane was incubated with anti-mouse/anti-rabbit monoclonal-alkaline phosphatase conjugated secondary antibody. The color was developed using BCIP and NBT as substrates. The relative band density was calculated with respect to VDAC, which was developed under the similar conditions using computerized densitometry system (Alpha Imager System, Alpha Innotech Corporation, San Leandro, CA, USA).

2.11. Statistical analysis

Data obtained from multiple experimental groups were compared using one-way analysis of variance, followed by Newman–Keuls post hoc test. However, Student's t-test was used for comparisons where only two groups were involved. The data are expressed in means ± standard error of means (SEM). The difference was considered statistically significant, when 'p' value was <0.05.

3. Results

3.1. Dopamine content

MPTP or combined maneb and paraquat depleted dopamine content in the striatum. While levodopa, MnTMPyP or minocycline significantly brought back maneb- and paraquat-mediated depletion in dopamine content, restoration in MPTP-mediated dopamine depletion was observed in the presence of levodopa or MnTMPyP but not in the presence of minocycline. Minocycline, levodopa or MnTMPyP alone did not alter dopamine content in the striatum (Fig. 1A).

3.2. Assessment of purity of the mitochondrial fraction and Mn-SOD protein expression

Significant SDH activity (241.8 ± 39.9, $p < 0.05$) and insignificant LDH activity (18.5 ± 8.0, $p < 0.001$) were noted in the mitochondrial fraction as compared with the total homogenate (considered 100% for all independent sets of experiments in case of SDH or LDH). On the contrary, significant LDH activity (309.4 ± 59.6, $p < 0.05$) and insignificant SDH activity (26.4 ± 11.3, $p < 0.01$) in the cytoplasmic fraction were observed as compared with the total homogenate. Such observations showed that the fractionation cleanly separated the mitochondria from the nigrostriatal tissues. MPTP or combined maneb and paraquat reduced the expression of Mn-SOD protein in the mitochondrial fraction of the nigrostriatal tissues. The expression level was significantly restored towards normal level in the animals treated with MnTMPyP or levodopa along with MPTP or maneb and paraquat.

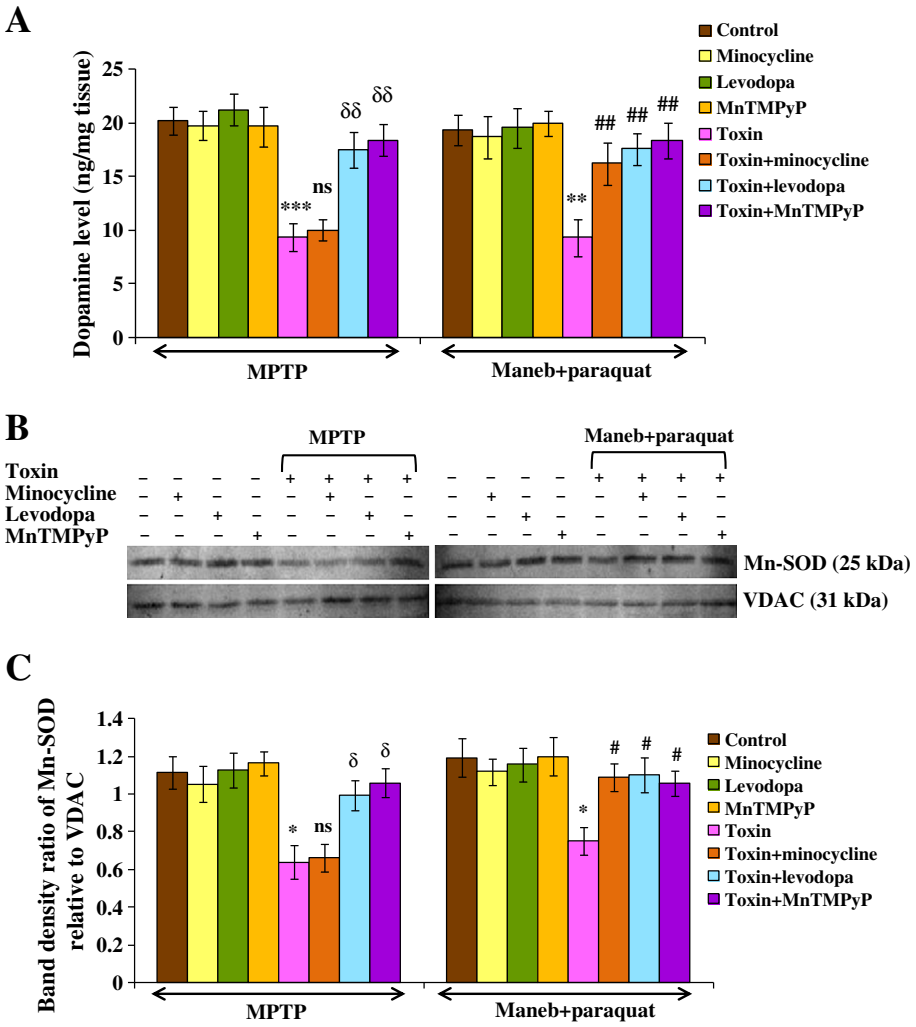

Fig. 1. Effects of MPTP and maneb and paraquat on the striatal dopamine content and Mn-SOD expression in the nigrostriatal tissues in the presence or absence of levodopa, minocycline or MnTMPyP. Bar diagram representing the dopamine level in the striatum of MPTP- and maneb- and paraquat-treated groups with or without levodopa, minocycline and MnTMPyP treatment is shown in panel (A). Western blots of Mn-SOD in MPTP and maneb- and paraquat-treated groups with or without levodopa, minocycline and MnTMPyP treatment are shown in panel (B) while bar diagram representing the relative band density ratio of Mn-SOD and VDAC in MPTP- and maneb- and paraquat-treated groups with or without levodopa, minocycline and MnTMPyP treatment is depicted in panel (C). The values are calculated as means ± SEM (n = 3 independent experiments). Significant changes are expressed as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in comparison with controls, δ ($p < 0.05$) and δδ ($p < 0.01$) in comparison with MPTP-treated group [F = 10.680 and 6.259 in case of dopamine level and Mn-SOD expression, respectively and df (among groups) = 7 and df (within groups) = 16] and # ($p < 0.05$) and ## ($p < 0.01$) in comparison with maneb- and paraquat-treated group [F = 4.299 and 2.875 in case of dopamine level and Mn-SOD expression, respectively and df (among groups) = 7 and df (within groups) = 16]. The insignificant change with respect to MPTP-treated group is represented as 'ns' and the group "Toxin" refers to the animals treated with either MPTP or maneb and paraquat.

Minocycline restored the expression of Mn-SOD significantly only in maneb- and paraquat-treated group (Fig. 1B and C).

3.3. TH-immunoreactivity

MPTP or maneb and paraquat in combination attenuated the number of TH-positive neurons in the substantia nigra. Minocycline, levodopa or MnTMPyP alone did not alter the level of TH-immunoreactivity. Levodopa or MnTMPyP significantly reinstated the MPTP- or maneb- and paraquat-induced changes in the number of TH-positive neurons. Minocycline rescued maneb- and paraquat-induced decrease in the number of TH-positive neurons while it did not produce significant change in MPTP-treated animals (Fig. 2).

3.4. Microglial activation

MPTP or maneb and paraquat in combination augmented the number of activated microglia in the substantia nigra. Minocycline, MnTMPyP or levodopa alone did not alter the number of activated microglia. Minocycline, MnTMPyP or levodopa reduced the number of activated microglia in the substantia nigra of MPTP- or maneb- and paraquat-treated animals. However, the reduction was more pronounced in minocycline- or MnTMPyP-treated groups (Fig. 3).

3.5. Mitochondrial complex activity

Mitochondrial complex I (NADH: ubiquinone reductase or NADH dehydrogenase) activity was significantly reduced by MPTP or maneb and paraquat. MPTP-induced inhibition in the mitochondrial complex I activity was significantly rescued by MnTMPyP or levodopa but not by minocycline. However, maneb and paraquat-mediated alteration was significantly restored by all the three agents (Table 1).

Trifling and statistically insignificant reduction in the mitochondrial complex III (coenzyme Q: cytochrome c oxidoreductase or cytochrome b–c1 complex) activity was observed in maneb- and paraquat-treated animals. Mitochondrial complex III activity in the

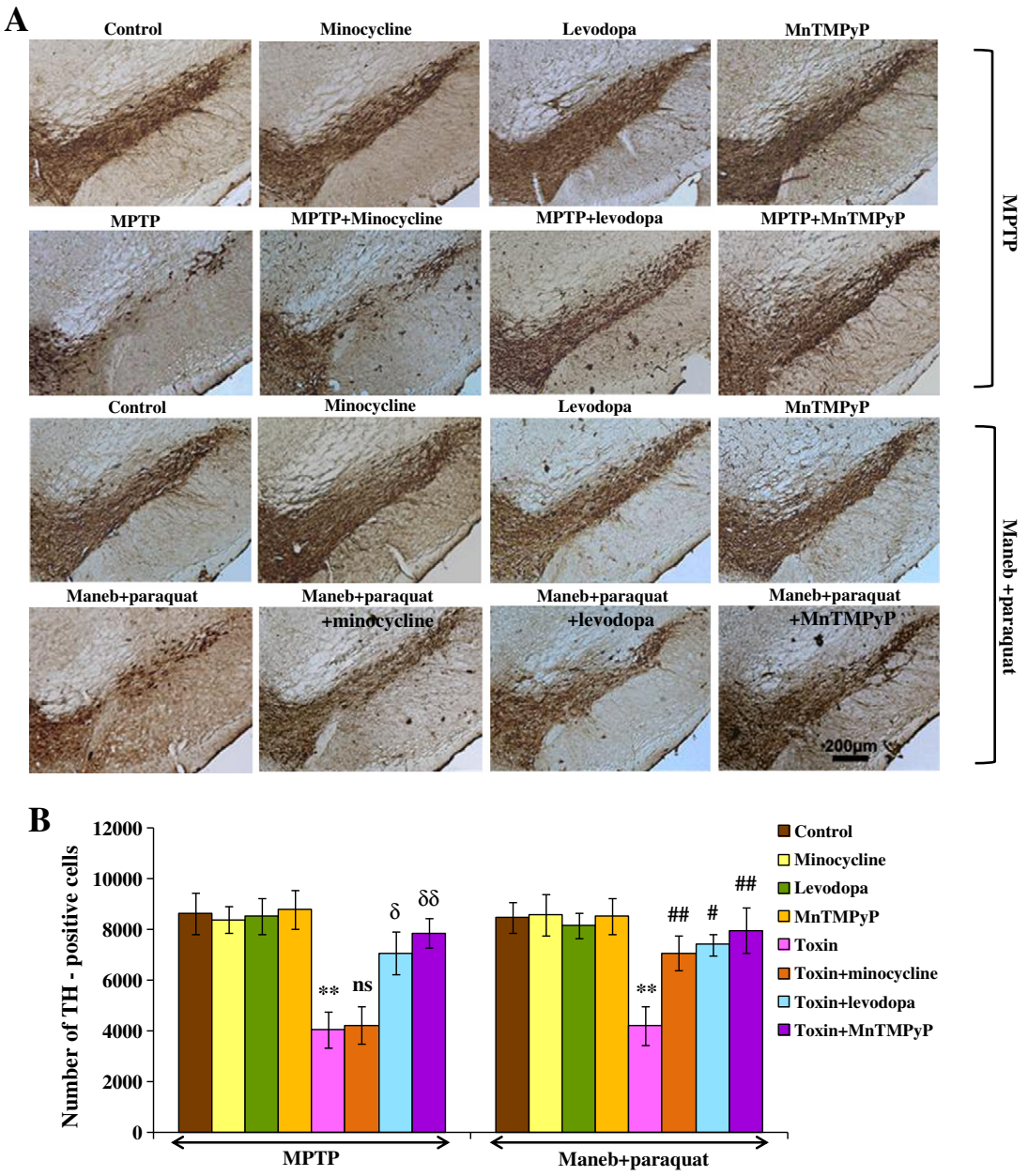

Fig. 2. Effects of MPTP and maneb and paraquat on TH-immunoreactivity in the substantia nigra in the presence or absence of levodopa, minocycline and MnTMPyP. The images taken employing objective lenses of 5× magnification power (total magnification: 50×) are shown in panel (A) while the number of TH-positive neurons is shown in the form of bar diagram in panel (B). The values are calculated as means ± SEM (n = 3 independent experiments). Significant changes are expressed as ** ($p < 0.01$) in comparison with controls, δ ($p < 0.05$) and δδ ($p < 0.01$) in comparison with MPTP-treated group [$F = 7.351$ and df (among groups) = 7 and df (within groups) = 16] and # ($p < 0.05$) and ## ($p < 0.01$) in comparison with maneb- and paraquat-treated group [$F = 4.471$ and df (among groups) = 7 and df (within groups) = 16]. The insignificant change with respect to MPTP-treated group is represented as 'ns' and the group "Toxin" refers to the animals treated with either MPTP or maneb and paraquat.

nigrostriatal tissues was not altered in MPTP treated animals. Similarly, no significant change in the mitochondrial complex III activity was observed in the remaining treated groups (data not shown).

3.6. 2-D PAGE and mass spectrometry

The proteins that displayed statistically significant changes in abundance of at least 1.4 fold were identified by MALDI-TOF/TOF mass spectrometry. Twelve protein spots selected from MPTP-treated group (Fig. 4 and Table 2) on the basis of the criteria mentioned above were identified by MALDI-TOF/TOF. Up-regulated proteins were identified as ATP synthase β, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (DLST), IDH3α, γ-enolase, α-enolase and tubulin α-1A chain while down-regulated proteins were identified as cytochrome c oxidase subunit 5a (COX 5a) preprotein, stathmin, dihydropyrimidinase-related protein-2 (DRP-2), septin-5, Prx3 and Prx2. Similarly, fourteen protein spots from maneb- and paraquat-treated group (Fig 5 and Table 3), which fulfilled the selection criteria, were identified by MALDI-TOF/TOF. The proteins, which were up-regulated as compared with respective controls, were identified as complex III subunit 1, DLST, IDH3α, pyruvate dehydrogenase E1 component subunit α (PDH E1α), γ-enolase, α-enolase, tubulin α-1A chain and actin-1 and proteins, which were down-regulated, were identified as ATP synthase α, COX 5a preprotein,

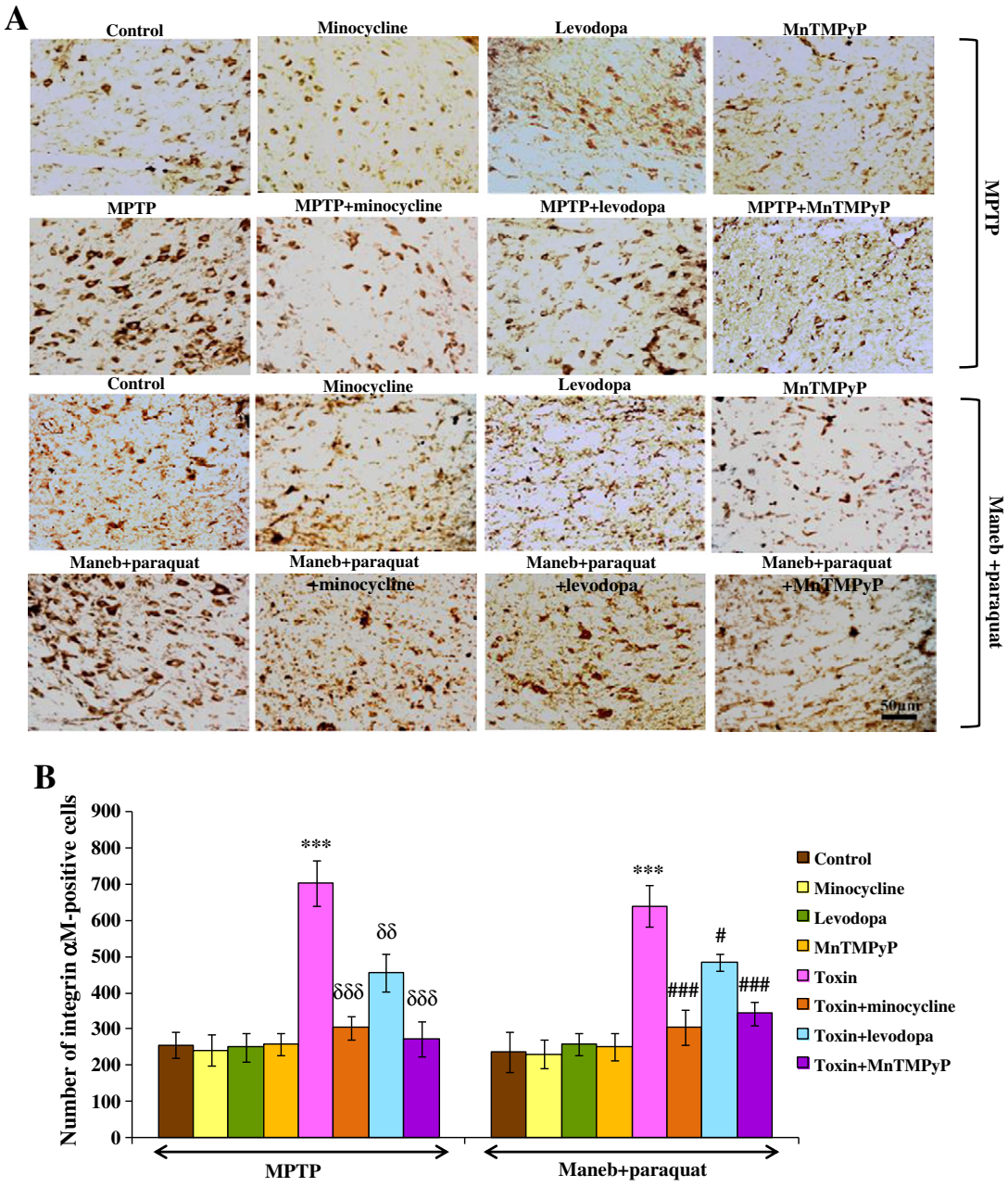

Fig. 3. Effects of MPTP and maneb and paraquat on integrin αM-positivity in the substantia nigra in the presence or absence of levodopa, minocycline or MnTMPyP. The images taken employing the objective lenses of 20× magnification power (total magnification: 200×) are shown in panel (A), however, the number of activated microglia is shown in the form of bar diagram in panel (B). The values are calculated as means ± SEM (n = 3 independent experiments). Significant changes are expressed as *** ($p < 0.001$) in comparison with controls, δδ ($p < 0.01$) and δδδ ($p < 0.001$) in comparison with MPTP-treated group [F = 13.480 and df (among groups) = 7 and df (within groups) = 16] and # ($p < 0.05$) and ### ($p < 0.001$) in comparison with maneb- and paraquat-treated group [F = 11.820 and df (among groups) = 7 and df (within groups) = 16]. The group "Toxin" refers to the animals treated with either MPTP or maneb and paraquat.

Table 1
Statistics of the mitochondrial complex I activity (expressed in terms of percentage of controls) of the nigrostriatal tissues. The values are calculated as means ± SEM (n = 3 independent experiments). Significant changes are expressed as ** ($p < 0.01$) and *** ($p < 0.001$) in comparison with controls, δδ ($p < 0.01$) and δδδ ($p < 0.001$) in comparison with MPTP [F = 19.880 and df (among groups) = 7 and df (within groups) = 16] and # ($p < 0.05$) and ## ($p < 0.01$) in comparison with maneb- and paraquat-treated groups [F = 6.054 and df (among groups) = 7 and df (within groups) = 16]. The insignificant change with respect to MPTP-treated group is represented as 'ns'.

MPTP group	Complex I specific activity (% of control)	Maneb + paraquat group	Complex I specific activity (% of control)
Control	100 ± 0.0	Control	100 ± 0.0
Minocycline	99.7 ± 4.3	Minocycline	97.9 ± 5.9
Levodopa	98.2 ± 3.4	Levodopa	99.7 ± 6.1
MnTMPyP	97.2 ± 4.5	MnTMPyP	99.8 ± 4.0
MPTP	$61 \pm 2.9^{***}$	Maneb + paraquat	$66.2 \pm 5.3^{**}$
MPTP + minocycline	65.8 ± 4.0^{ns}	Maneb + paraquat + minocycline	$82.5 \pm 4.7^{\#}$
MPTP + levodopa	$83.2 \pm 2.9^{\delta\delta}$	Maneb + paraquat + levodopa	$84.1 \pm 4.1^{\#}$
MPTP + MnTMPyP	$90 \pm 3.4^{\delta\delta\delta}$	Maneb + paraquat + MnTMPyP	$91.8 \pm 5.8^{\#\#}$

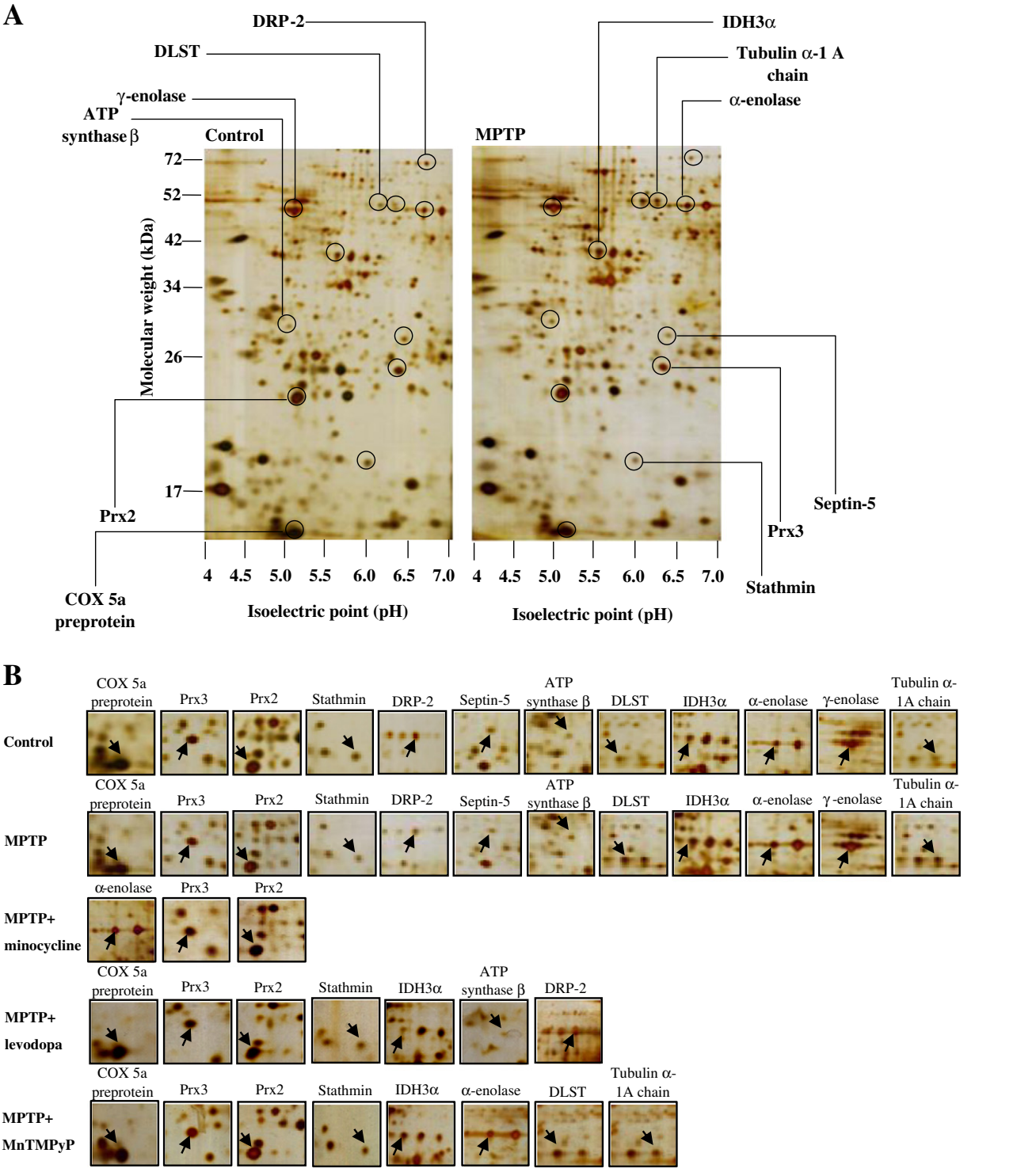

Fig. 4. Effect of MPTP on the nigrostriatal mitochondrial proteome profile in the presence or absence of levodopa, minocycline or MnTMPyP. 2-D gel electrophoretograms and differentially expressed proteins in MPTP-treated groups in comparison with control are shown in panel (A) and alterations in the expression of the proteins in the presence of levodopa, minocycline or MnTMPyP are shown in panel (B).

phosphatidylethanolamine-binding protein 1 (PEBP1), stathmin, Prx3 and ubiquitin carboxyl-terminal hydrolase isozyme-L1 (UCH-L1). Eight proteins-DLST, IDH3α, γ-enolase, α-enolase, tubulin α-1A chain and COX 5a preprotein, stathmin and Prx3 were common between maneb- and paraquat- and MPTP-treated groups in terms of the differential expression patterns. DRP-2, Prx2, septin-5 and ATP synthase β were differentially regulated only in MPTP-treated animals while PEBP1, UCH-L1, ATP synthase α, complex III subunit 1, PDH E1α and actin-1 were altered in maneb- and paraquat-treated animals. Differentially expressed proteins were found to be associated with the

Table 2
Fold changes of differentially expressed proteins in MPTP-treated animals in the presence or absence of levodopa, minocycline or MnTMPyP. The values are calculated as means ± SEM (n = 3 independent experiments). Significant changes are expressed as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in comparison with controls and δ ($p < 0.05$), δδ ($p < 0.01$) and δδδ ($p < 0.001$) in comparison with MPTP-treated groups. The insignificant changes with respect to MPTP-treated group are represented as 'ns' (F-value for each protein is shown in table while df values among groups and within groups are 7 and 16, respectively). The fold changes are calculated with respect to controls (considering 1 in all independent set of experiments); therefore, there is no SEM in controls.

Protein name	Control	Minocycline	Levodopa	MnTMPyP	MPTP + minocycline	MPTP	MPTP + levodopa	MPTP + MnTMPyP	F-value
COX 5a preprotein	1.00 ± 0.00	0.97 ± 0.05	0.95 ± 0.11	0.93 ± 0.04	0.61 ± 0.04**	0.59 ± 0.01ns	0.87 ± 0.06δ	0.85 ± 0.01 δδ	8.182
Prx3	1.00 ± 0.00	0.94 ± 0.10	0.95 ± 0.06	0.99 ± 0.10	0.48 ± 0.05**	0.77 ± 0.06 δ	0.74 ± 0.08 δ	0.84 ± 0.02 δ	6.016
Prx2	1.00 ± 0.00	0.93 ± 0.09	0.97 ± 0.02	0.99 ± 0.06	0.67 ± 0.04*	0.88 ± 0.05 δ	0.90 ± 0.02 δ	0.89 ± 0.06 δ	3.798
Stathmin	1.00 ± 0.00	0.97 ± 0.05	0.92 ± 0.03	1.02 ± 0.07	0.52 ± 0.07***	0.57 ± 0.04 ns	0.81 ± 0.04 δδ	0.89 ± 0.07 δδ	12.29
Septin-5	1.00 ± 0.00	0.93 ± 0.08	0.93 ± 0.08	0.98 ± 0.08	0.45 ± 0.12*	0.41 ± 0.04 ns	0.52 ± 0.13 ns	0.59 ± 0.12 ns	7.127
DRP-2	1.00 ± 0.00	0.96 ± 0.06	1.02 ± 0.04	1.00 ± 0.08	0.62 ± 0.07**	0.60 ± 0.03 ns	0.89 ± 0.06 δ	0.69 ± 0.09 ns	7.792
ATP synthase β	1.00 ± 0.00	0.98 ± 0.09	0.96 ± 0.03	1.01 ± 0.10	2.01 ± 0.19***	1.87 ± 0.03 ns	1.27 ± 0.13 δδδ	1.69 ± 0.09 ns	18.05
DLST	1.00 ± 0.00	1.09 ± 0.17	1.06 ± 0.16	1.08 ± 0.07	1.93 ± 0.29**	1.79 ± 0.08 ns	1.90 ± 0.10 ns	1.18 ± 0.02 δδ	8.292
IDH3α	1.00 ± 0.00	0.98 ± 0.08	1.01 ± 0.11	0.97 ± 0.09	2.06 ± 0.24**	1.92 ± 0.28 ns	1.10 ± 0.07 δδ	1.21 ± 0.15 δδ	7.884
α-Enolase	1.00 ± 0.00	1.00 ± 0.03	1.00 ± 0.04	1.02 ± 0.07	1.56 ± 0.10**	1.18 ± 0.11 δ	1.52 ± 0.18 ns	1.09 ± 0.04 δ	6.546
γ-Enolase	1.00 ± 0.00	1.00 ± 0.12	1.05 ± 0.12	1.02 ± 0.12	1.64 ± 0.06*	1.60 ± 0.19 ns	1.47 ± 0.08 ns	1.50 ± 0.10 ns	6.195
Tubulin α-1A chain	1.00 ± 0.00	1.01 ± 0.07	0.99 ± 0.07	1.05 ± 0.08	2.06 ± 0.25***	1.86 ± 0.09 ns	1.97 ± 0.13 ns	1.27 ± 0.20 δδ	12.04

mitochondrial electron transport chain, glucose metabolism, antioxidant function, neuronal growth or with synaptic function, as inferred from the literature.

Minocycline restored the expressions of three proteins in MPTP-treated group (Fig. 4 and Table 2) and seven proteins in maneb- and paraquat-treated groups (Fig. 5 and Table 3). Minocycline reduced the MPTP-mediated up-regulation in the expression of α-enolase and restored the down-regulation of Prx3 and Prx2 towards controls with varied levels of statistical significances. While minocycline reduced the expressions of DLST, tubulin alpha-1A chain and α-enolase, it augmented Prx3, COX 5a preprotein, ATP synthase α and stathmin expressions in maneb- and paraquat-treated animals. Moreover, the restoration in the expression patterns of Prx3 and α-enolase was common in both MPTP- and maneb- and paraquat-treated groups.

Levodopa altered the expressions of seven proteins in MPTP and eight proteins in maneb- and paraquat-treated groups towards normal levels (Figs. 4 and 5 and Tables 2 and 3). Levodopa increased the expressions of Prx3, Prx2, stathmin, DRP-2 and COX 5a preprotein, while it reduced the expressions of IDH3α and ATP synthase β in MPTP-treated animals as compared with MPTP alone treated animals. Similarly, levodopa increased the expressions of Prx3, PEBP1, stathmin, COX 5a preprotein and decreased the expressions of IDH3α, complex III subunit 1, tubulin α-1A chain and PDH E1α in maneb- and paraquat-treated group. Common restoration trend for Prx3, stathmin, IDH3α and COX 5a preprotein expressions was observed in maneb- and paraquat- as well as MPTP-treated groups.

MnTMPyP restored the expressions of eight differentially expressed proteins in maneb- and paraquat- and MPTP-treated groups (Figs. 4 and 5). Administration of MnTMPyP in MPTP-treated animals increased the expressions of Prx3, Prx2, stathmin and COX 5a preprotein and reduced the expressions of IDH3α, α-enolase, DLST and tubulin α-1A chain. Similarly, MnTMPyP increased the expressions of Prx3, ATP synthase α, stathmin, COX 5a preprotein and reduced the expressions of complex III subunit 1, IDH3α, DLST and tubulin α-1A chain in maneb- and paraquat-treated animals. Restoration in the expression patterns of Prx3, stathmin, COX 5a preprotein, IDH3α, DLST and tubulin α-1A chain by MnTMPyP exhibited the similar trend in both maneb- and paraquat- and MPTP-treated groups (Figs. 4 and 5 and Tables 2 and 3).

3.7. Western blotting of IDH3α, stathmin and Prx3

The western blot analyses of stathmin, IDH3α and Prx3 were done to validate the expression patterns of the proteins obtained from 2-D PAGE. Prx3, a mitochondrial antioxidant and stathmin, a microtubule-destabilizing protein, showed down-regulation while IDH3α, a protein involved in citric acid cycle, showed up-regulation in MPTP as well as maneb- and paraquat-treated animals, as also observed in 2-D PAGE. IDH3α, Prx3 and stathmin expressions were restored, when toxicant(s)-treated animals were also challenged with levodopa or MnTMPyP while no significant alteration was noticed in the expression of stathmin in animals treated with minocycline and MPTP (Fig. 6). The expression of IDH3α remained unchanged in minocycline treated groups that were also exposed to MPTP or maneb and paraquat. The western blot images presented in the figures represent the pictures of a representative set selected from three to four independent sets of experiments rather than the best images of specific protein from different sets while the bar diagrams are the averages of all the experiments; therefore, some minor deviations could be noted while comparing the images with bars.

4. Discussion

MPTP and maneb and paraquat in combination reduced the number of TH-positive neurons and depleted the level of dopamine in the dorsal striatum. Levodopa and MnTMPyP significantly rescued the toxicants-mediated dopaminergic neuronal loss and dopamine depletion in both models as also reported elsewhere [14,15,19,20,36]. Statistically insignificant increase in the striatal dopamine level was observed in levodopa alone treated animals. This is in accordance with many reports, which have shown lack of significant change in the striatal dopamine content after levodopa administration in controls [37,38]. While minocycline significantly ameliorated the loss of TH-positive neurons and dopamine depletion in maneb- and paraquat-treated animals [39], the changes were insignificant in MPTP-treated animals in this study, as observed in earlier study [40]. This could be due to inability of minocycline to restore the MPTP-induced complex I inhibition and decreased Mn-SOD level. The results provide a clue that superoxide radical could act as an important mediator in MPTP-induced neurodegeneration. Reduction in microglial activation reflects the anti-inflammatory nature of minocycline, levodopa or MnTMPyP against MPTP- or maneb- and paraquat-induced neurodegeneration [12,21,41]. The inhibition of microglial activation observed with minocycline in animals treated with MPTP reflects its anti-inflammatory property, which could not be correlated with overall neurodegeneration [40]. We do not have direct experimental evidence whether differences in the efficacy of particular treatments against both models reflect mechanistic distinctions or not. It could be mechanistic distinctions rather than trivial explanations since animals were treated with levodopa, minocycline or MnTMPyP 2 h prior to respective neurotoxin and the extent of neurodegeneration observed at the end in both the models was comparable. Additionally, under the current paradigms, the drugs were given 2 h prior to neurotoxin, which do not mimic a real-life situation

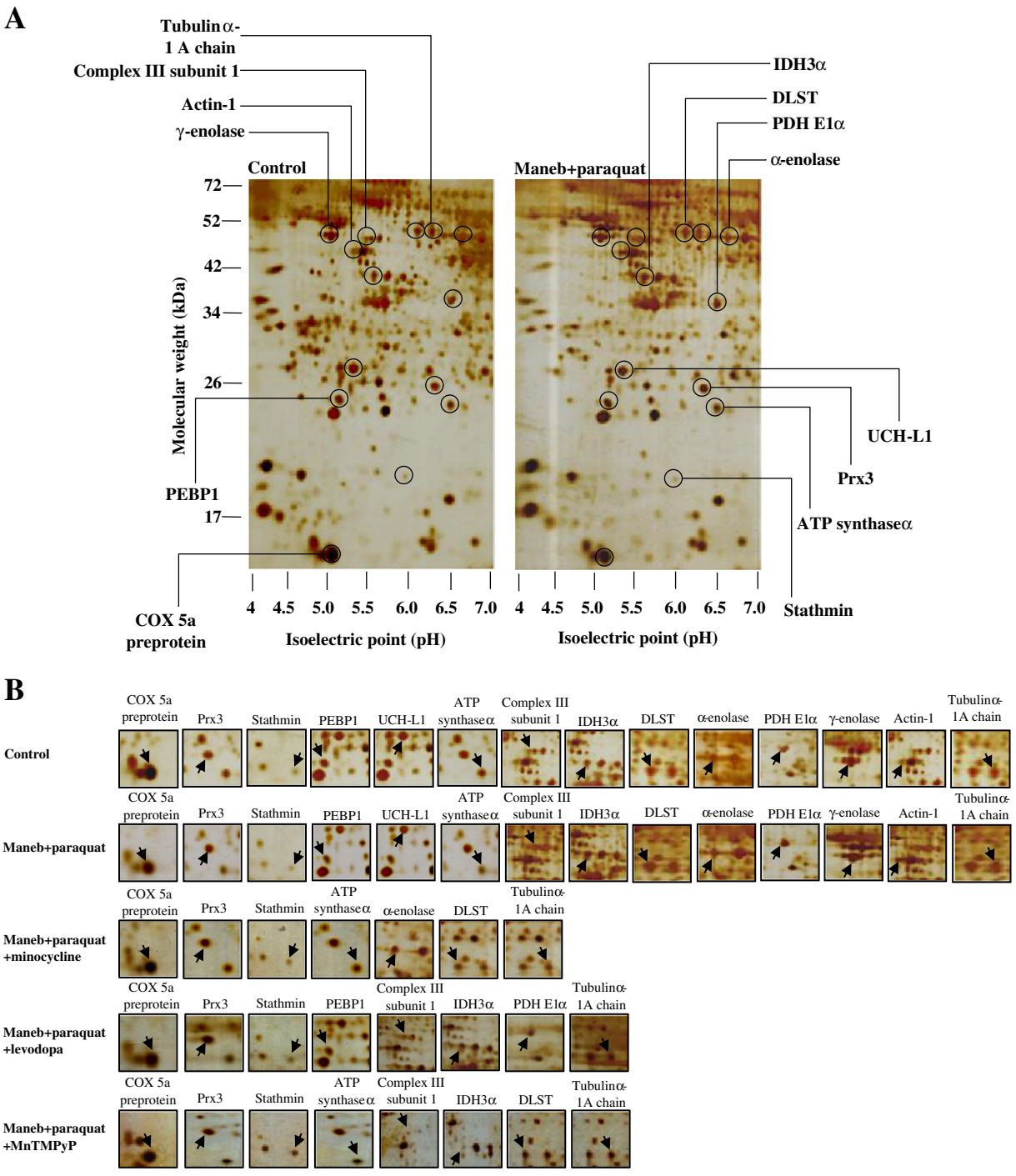

Fig. 5. Effect of maneb and paraquat on the nigrostriatal mitochondrial proteome profile in the presence or absence of levodopa, minocycline or MnTMPyP. 2-D gel electrophoretograms and differentially expressed proteins in maneb- and paraquat-treated groups in comparison with controls are shown in panel (A) and modulations in the expression levels of differentially expressed proteins in the presence of levodopa, minocycline or MnTMPyP are shown in panel (B).

where the cellular insult (be it a mutation or environmental factor) is likely to be present for a number of years before patients are first diagnosed and treated.

Negligible LDH activity in the mitochondrial fraction exhibited the purity of the mitochondrial fraction. MPTP or maneb and paraquat significantly inhibited complex I but not complex III in the study. Unaltered complex III in MPTP-treated animals is in accordance with an earlier finding [5] while no change in the activity of complex III in maneb- and paraquat-treated animals reflects that maneb and paraquat cause toxicity differently as compared with maneb alone or maneb-induced toxicity could also act through other targets in addition to the mitochondrial electron transport chain [42]. Complex I inhibition led to an incomplete reduction of oxygen and enhanced superoxide radical production, which was reflected from the reduced mitochondrial Mn-SOD expression. Nitric oxide generated by the activated microglia could inactivate Mn-SOD [43]. This could be correlated with the reports, which have shown that the mitochondrial antioxidant defense system gets impaired in MPTP- or paraquat-induced

Table 3
Fold changes of differentially expressed proteins in maneb- and paraquat-treated animals in the presence or absence of levodopa, minocycline or MnTMPyP. The values are calculated as means ± SEM (n = 3 independent experiments). Significant changes are expressed as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) in comparison with controls and # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$) in comparison with maneb- and paraquat-treated groups. The insignificant changes with respect to maneb- and paraquat-treated group are represented as 'ns' (F-value for each protein is shown in table while df values among groups and within groups are 7 and 16, respectively). The fold changes are calculated with respect to controls (considering 1 in all independent experiments); therefore, SEM value is 0 in controls.

Protein name	Control	Minocycline	Levodopa	MnTMPyP	Maneb + paraquat	Maneb + paraquat + minocycline	Maneb + paraquat + levodopa	Maneb + paraquat + MnTMPyP	F-value
COX 5a preprotein	1.00 ± 0.00	0.96 ± 0.08	0.96 ± 0.06	0.97 ± 0.04	0.62 ± 0.06**	0.82 ± 0.01#	0.92 ± 0.05##	0.89 ± 0.04##	5.207
Prx3	1.00 ± 0.00	1.01 ± 0.08	0.91 ± 0.07	0.97 ± 0.02	0.46 ± 0.08***	0.77 ± 0.07#	0.74 ± 0.05#	0.69 ± 0.10#	7.865
PEBP1	1.00 ± 0.00	0.99 ± 0.06	0.93 ± 0.02	0.98 ± 0.06	0.59 ± 0.07**	0.61 ± 0.09 ns	0.85 ± 0.04#	0.63 ± 0.03 ns	9.474
Stathmin	1.00 ± 0.00	1.03 ± 0.03	0.97 ± 0.13	1.03 ± 0.06	0.50 ± 0.07**	0.92 ± 0.10##	0.84 ± 0.06##	1.03 ± 0.09##	5.058
UCH-L1	1.00 ± 0.00	0.95 ± 0.06	0.98 ± 0.03	0.98 ± 0.01	0.70 ± 0.07*	0.78 ± 0.04 ns	0.82 ± 0.10 ns	0.69 ± 0.01 ns	5.811
ATP synthase α	1.00 ± 0.00	0.99 ± 0.07	0.95 ± 0.09	1.01 ± 0.03	0.55 ± 0.10*	0.91 ± 0.04#	0.60 ± 0.08 ns	0.88 ± 0.11#	5.493
Complex III subunit 1	1.00 ± 0.00	1.03 ± 0.23	1.11 ± 0.30	0.94 ± 0.10	2.64 ± 0.20***	2.44 ± 0.25 ns	1.64 ± 0.26##	1.44 ± 0.13#	10.06
PDH E1α	1.00 ± 0.00	1.02 ± 0.08	0.93 ± 0.007	0.98 ± 0.07	1.54 ± 0.11*	1.51 ± 0.14 ns	1.14 ± 0.02#	1.58 ± 0.17 ns	7.936
DLST	1.00 ± 0.00	0.96 ± 0.29	0.98 ± 0.12	0.92 ± 0.13	2.29 ± 0.12***	1.41 ± 0.16##	2.03 ± 0.11 ns	1.46 ± 0.19##	10.44
IDH3α	1.00 ± 0.00	0.98 ± 0.13	0.92 ± 0.11	0.93 ± 0.13	1.84 ± 0.23**	1.52 ± 0.25 ns	1.16 ± 0.08#	1.01 ± 0.08##	4.748
α-Enolase	1.00 ± 0.00	1.06 ± 0.11	1.03 ± 0.12	0.98 ± 0.01	1.49 ± 0.04**	1.19 ± 0.02#	1.53 ± 0.08 ns	1.55 ± 0.14 ns	8.473
γ-Enolase	1.00 ± 0.00	0.95 ± 0.07	0.99 ± 0.15	1.00 ± 0.02	1.74 ± 0.22**	1.58 ± 0.16 ns	1.67 ± 0.07 ns	1.79 ± 0.16 ns	8.628
Tubulin α-1A chain	1.00 ± 0.00	0.97 ± 0.14	0.94 ± 0.17	1.16 ± 0.13	1.82 ± 0.09**	1.19 ± 0.02#	1.42 ± 0.17#	1.26 ± 0.15#	4.962
Actin-1	1.00 ± 0.00	1.17 ± 0.20	1.15 ± 0.13	1.01 ± 0.27	2.64 ± 0.22**	2.48 ± 0.29 ns	2.54 ± 0.28 ns	2.21 ± 0.22 ns	11.41

Parkinsonism [17,42]. Minocycline restored the mitochondrial complex I inhibition and Mn-SOD level in maneb- and paraquat-treated animals thereby attenuating the generation of reactive oxygen species, which reflects the involvement of mitochondria in minocycline-induced neuroprotection [23]. Restoration of complex I activity and Mn-SOD expression by levodopa or MnTMPyP indicate that both offer neuroprotection at the mitochondrial level by reducing oxidative stress generated by MPTP or maneb and paraquat combined. Conflicting reports are available in literature regarding the effect of levodopa on the complex I activity. In this study, levodopa was found to increase the complex I activity, when given with MPTP or maneb and paraquat, however, it did not alter the complex I activity, when administered alone. This is in accordance with the previous reports, which have demonstrated the improvement in motor dysfunctions by levodopa in mice having mitochondrial defects [22]. MnTMPyP-mediated increase in complex I activity could also be explained on the basis of a report, which has shown the reduced complex I activity in heterozygous Mn-SOD knockout mice [44]. VDAC1 was used as a protein loading control in the western blot analyses. While a few studies, including a proteomics study, which employed MPTP-treated neuroblastoma cells [24], have shown an alteration in VDAC 1 protein expression after MPTP treatment, no statistically significant changes were observed in any of the treated groups, including MPTP, in the current study, as compared with controls. This is in accordance with many reports [45–48], which have also used VDAC1 as a loading control and did not see significant change in VDAC protein expression after MPTP or paraquat exposure.

The proteome profile of MPTP and maneb and paraquat was compared since the former is the most widely used mice model while latter is environmentally relevant and a chronic PD model. Down-regulation of COX 5a preprotein, a component of mitochondrial complex IV, in MPTP- and maneb- and paraquat-treated groups is in accordance with a previous study [49]. Up-regulation of ATP synthase β and down-regulation of ATP synthase α, components of complex V after MPTP and maneb and paraquat treatment, respectively, reveal that both agents alter the mitochondrial integrity. Maneb and paraquat treatment increased the level of complex III subunit 1 protein, which is in accordance with a study conducted employing 6-OHDA model [50]. Altered level of subunits of the respiratory chain complexes in MPTP- and maneb- and paraquat-treated animals reflects that the inhibition of complex I could disturb the expression of various proteins of the respiratory chain, which may lead to mitochondrial damage and subsequent neuronal death. Up-regulation of DLST, IDH3α, γ-enolase and α-enolase in MPTP- and maneb- and paraquat-treated groups and PDH E1α in only latter group indicate that in spite of common targets, a few variable steps are also targeted. Altered levels of the IDH3α, γ-enolase and α-enolase are in accordance with the previous reports related to PD or other neurological disorders [51,52]. Mutations in a gene that encodes DLST subunit of α-ketoglutarate dehydrogenase complex and another gene, which encodes E1α subunit of pyruvate dehydrogenase complex, have been reported to be associated with increased risk of PD [53,54]. Although decreased expression of α-enolase in total cellular proteins is reported [29], the level was increased in the mitochondrial fraction in this study. Toxic insult of MPTP or maneb and paraquat could increase an interaction of α-enolase with the mitochondrial membrane and thereby an increased level in the mitochondrial fraction [51]. Decreased expression of Prx3 in MPTP- or maneb- and paraquat-treated group and Prx2 in MPTP-treated animals indicate that toxins could impair the mitochondrial and cellular antioxidant defense systems. Reduced level of Prx3 is reported in PD patients [55] while Prx2 is found down-regulated in MPTP-induced PD [56]. Stathmin destabilizes microtubule assembly, binds to tubulin and helps in intracellular organelle migration thus participates in neuronal growth [57]. Decreased stathmin and increased tubulin expressions in MPTP- and maneb- and paraquat-treated groups indicate the impaired mitochondrial translocation and neuronal growth. Down-regulation of stathmin is also reported in the cypermethrin-induced neurodegeneration [11]. Increased level of actin in response to maneb and paraquat could indicate occurrence of an interaction between mitochondria and cytoskeleton, which could have significantly hampered due to toxic insult. Down-regulation of DRP-2 in MPTP- and PEBP1 in maneb- and paraquat-treated group indicate possible defect in the neuronal repair and growth [58,59]. The decreased level of septin-5 in response to MPTP could lead to an accumulation of MPP^+ inside the synaptic vesicle and impaired vesicle transport and fusion [60]. Down-regulation of UCH-L1, which hydrolyses polyubiquitin to monoubiquitin, in maneb- and paraquat-treated animals, indicates the reduced accessibility of free ubiquitin for conjugation with proteins for degradation. This is supported by a recent study, which has shown the down-regulation of UCH-L1 gene in maneb- and paraquat-induced Parkinsonism [61]. Although stathmin, UCH-L1 and Prx2 are not localized in the mitochondria, their presence in the mitochondrial fraction raised the possibility of their interaction or translocation to the mitochondrial membrane, as reported in a few studies [57,62,63].

Up-regulation of α-enolase and DLST expressions in maneb- and paraquat-treated animals was reduced after minocycline treatment, which could be due to restoration in the expression level of ATP synthase α and COX 5a preprotein. The results further indicate that minocycline either directly or indirectly modifies the deleterious mitochondrial effects from toxin exposure [23]. Minocycline was able to restore the expression of stathmin and tubulin in maneb- and paraquat-treated animals reflecting its ability to partially improve the transport of intracellular organelles or pathway required for neuronal growth [64]. The increased expression of Prx3 and Prx2 in the animals treated with minocycline along with MPTP and Prx3 in maneb- and paraquat-treated animals reveals anti-oxidative role of minocycline [39] and could be associated with its ability to encounter the NADPH oxidase-mediated production of free radicals leading to reduced microglial activation. Inflammatory response to toxic insult is related to enhanced production of plasminogen by activated

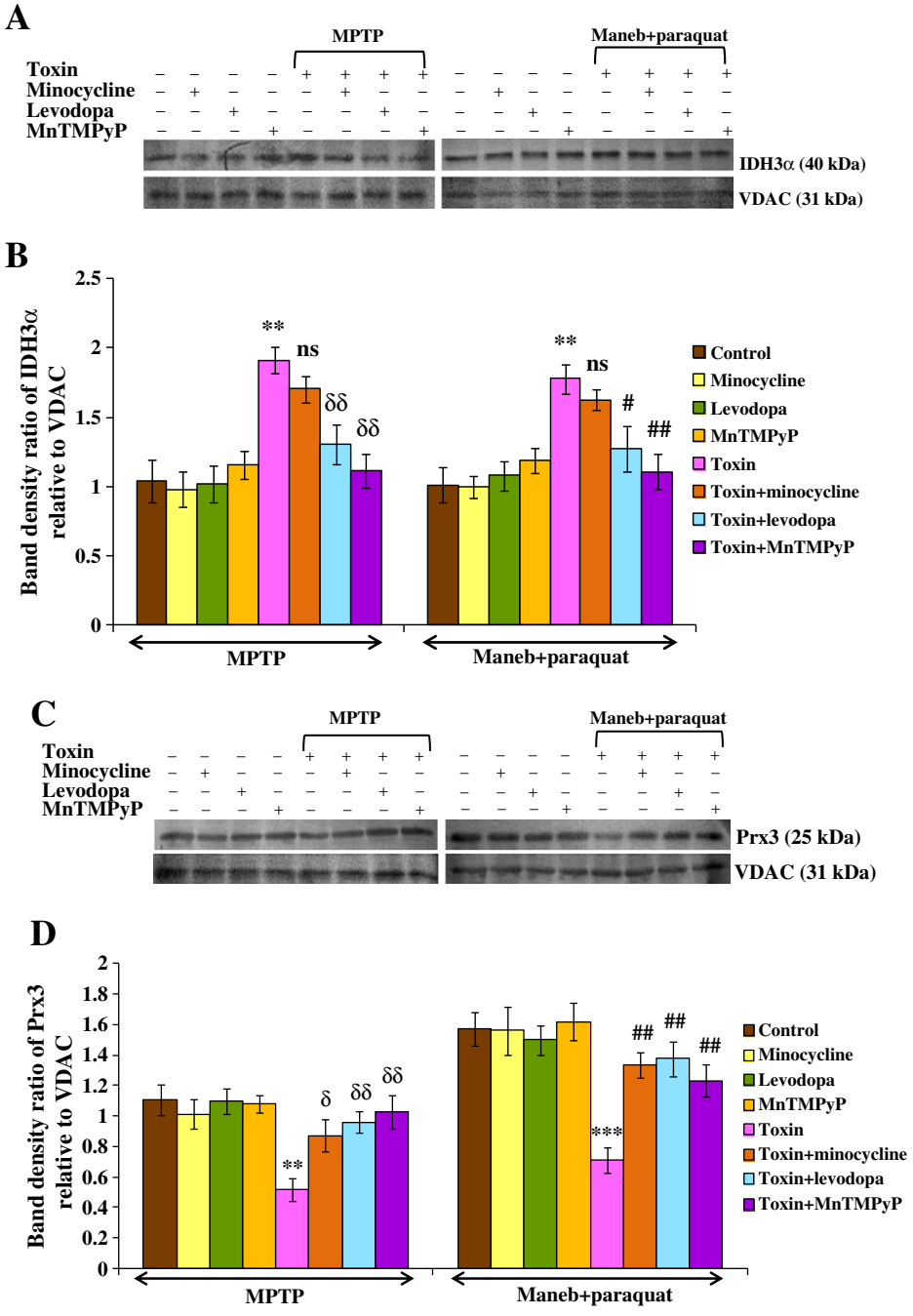

Fig. 6. Western blots of IDH3α, Prx3, stathmin and VDAC (a house keeping protein) of the nigrostriatal mitochondrial fraction of MPTP- or maneb- and paraquat-treated mice with or without levodopa, minocycline and MnTMPyP co-treatment are respectively shown in panels (A), (C) and (E). Bar diagrams representing the relative band density of IDH3α, Prx3, stathmin with respect to VDAC are shown in panels (B), (D) and (F), respectively. The values are expressed as means ± SEM (n = 3 independent experiments). Significant changes are expressed as ** ($p < 0.01$) and *** ($p < 0.001$) in comparison with controls, δ ($p < 0.05$) and δδ ($p < 0.01$) in comparison with MPTP [F = 7.882, 4.790 and 8.009 for IDH3α, Prx3 and stathmin expressions, respectively and df (among groups) = 7 and df (within groups) = 16] and # ($p < 0.05$) and ## ($p < 0.01$) in comparison with maneb- and paraquat-treated groups [F = 6.692, 6.965 and 4.333 for IDH3α, Prx3 and stathmin expressions, respectively and df (among groups) = 7 and df (within groups) = 16]. The insignificant changes with respect to MPTP- or maneb- and paraquat-treated group are represented as 'ns' and the group "Toxin" refers to the animals treated with either MPTP or maneb and paraquat.

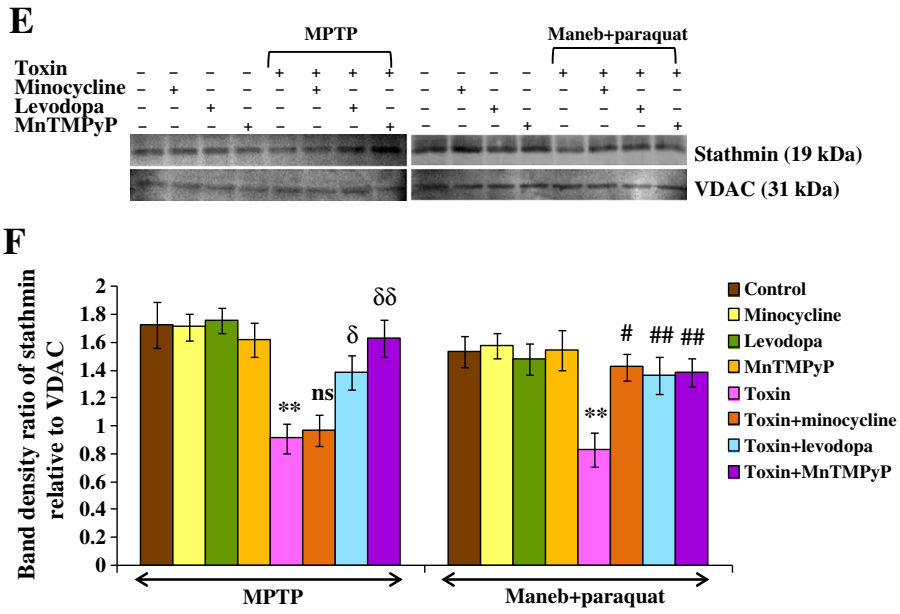

Fig. 6 (*continued*).

microglia [51]. Restoration in the increased level of α-enolase after minocycline treatment in MPTP- or maneb- and paraquat-treated animals could be correlated with decreased microglial activation in response to minocycline, as plasminogen binds with α-enolase [51]. Levodopa-induced blockade of complex I inhibition could restore COX 5a preprotein, complex III subunit 1, IDH3α and PDH E1α. The restoration of DRP-2, PEBP1, stathmin and tubulin by levodopa indicates that it could up-regulate neuronal growth and repair process, as reported elsewhere [13]. Levodopa increased the level of Prx3 and Prx2 showing that it can encounter oxidative stress [13]. MnTMPyP-induced restorations in COX 5a preprotein, complex III subunit 1, IDH3α, α-enolase, DLST, stathmin and tubulin could be due to its ability to encounter oxidative stress-induced changes in the expression of proteins by increasing Mn-SOD level. Decreased activity of electron transport chain enzymes in response to Mn-SOD deficiency is also reported in a study [44]. Overall, the study demonstrates that MPTP- and maneb- and paraquat-induced dopaminergic neurodegeneration are regulated through multiple routes. Neuroinflammation, mitochondrial dysfunction, microglial activation, and reduced neurotransmission could be the predominant pathways in both models while microglial activation-mediated alteration in the mitochondrial pathway could be critical in maneb- and paraquat-induced Parkinsonism. Levodopa and MnTMPyP protect against the changes induced by MPTP and combined maneb- and paraquat while minocycline offers neuroprotection mainly against combined maneb- and paraquat-induced Parkinsonism.

Conflict of interest

The authors declare that they have no conflict of interest.

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