Nigrostriatal proteomics of cypermethrin-induced dopaminergic neurodegeneration: Microglial activation dependent and independent regulations

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Abstract

The study aimed to identify the differentially expressed nigrostriatal proteins in cypermethrin-induced neurodegeneration and to investigate the role of microglial activation therein. Proteomic approaches were used to identify the differentially expressed proteins. Microglial activation, tyrosine hydroxylase immunoreactivity (TH-IR), dopamine content and neurobehavioral changes were measured according to the standard procedures. The expressions of α-internexin intermediate filament (α-IIF), ATP synthase D chain (ATP-SD), heat shock protein (Hsp)-70, truncated connexin-47, Hsp-60, mitogen activated protein kinase activated kinase-5, nicotinamide adenine dinucleotide (NADH2) dehydrogenase 24k chain precursor, platelet activating factor acetyl hydrolase 1b-α2 (PAF-AH1b-α2) and synaptosomal associated protein-25 (SNAP-25) were altered in the substantia nigra and NAD isocitrate dehydrogenase, phosphoethanolamine binding protein-1, prohibitin, protein disulfide isomerase-ER 60 protease, stathmin and ubiquitin conjugating enzyme in the striatum along with motor impairment, decreased dopamine and TH-IR and increased microglial activation after cypermethrin exposure. Minocycline restored α-IIF, ATP-SD chain, truncated connexin-47, Hsp-60, PAF-AH1b-α2, stathmin and SNAP-25 expressions, motor impairment, dopamine, TH-IR and microglial activation. The results suggest that cypermethrin produces microglial activation dependent and independent changes in the expression patterns of the nigrostriatal proteins leading to dopaminergic neurodegeneration.

Keywords Cypermethrin; proteomics; neurodegeneration; microglial activation
Introduction

Pyrethroids are one of the most commonly used classes of pesticides in agricultural and household formulations and account for one-fourth of the total insecticide market worldwide, despite well documented adverse effects (Casida and Quistad, 1998; Heudorf et al., 2004). Pesticides induce free radical generation leading to the nigrostriatal dopaminergic neurodegeneration, an important hallmark of Parkinson’s disease (PD) (Barbeau et al., 1987; Koller et al., 1990). Cypermethrin, a class II pyrethroid insecticide, crosses the blood-brain barrier, produces free radicals and induces oxidative damage in dopaminergic neurons of the nigrostriatal pathway leading to PD phenotype in experimental animals (Giray et al., 2001; Kale et al., 1999; Singh et al., 2010).

Proteomic approaches identify the differentially expressed proteins in sporadic and chemicals-induced PD and elucidate the roles of identified protein involved therein (Basso et al., 2004; Patel et al., 2007; Sinha et al., 2009; Srivastava et al., 2010; Tribl et al., 2009). Proteomic approaches offer widespread information on cellular physiology and its correlation with the expressed proteins because the information available at the transcriptional level does not always correlate with the translated proteins (Ideker et al., 2001). Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in combination with mass spectrometry (MS) and western blotting offers a comprehensive overview of cellular proteins involved in neurodegenerative disorders, including PD (LoPachin et al., 2003). Proteome analyses detect protein spots specific to a pathophysiological condition and possess potential to selectively and effectively differentiate neurological diseases (Finehout et al., 2007; Hu et al., 2007). The differential expressions of peroxiredoxin II, mitochondrial complex III, ATP synthase D (ATP-SD) chain, complexin-I, profilin, L-type calcium channel δ-subunit, fatty acid binding protein, ferritin H, a few isoforms
of glutathione-S-transferase and glial fibrillary acidic protein have been reported in the substantia nigra of PD patients (Basso et al., 2004; Werner et al., 2008). Several proteins, which include, superoxide dismutase, dimethylarginine dimethylaminohydrolase 1, α-synuclein, ubiquitin-conjugating enzyme, statmin 1, calcineurin B, cystatin B, subunit of mitochondrial H-ATP synthase, ATP-SD chain, mitochondrial nicotinamide adenine dinucleotide (NADH2) dehydrogenase (ubiquinone), glia maturation factor, α-enolase, complexin-I and lipid binding protein, etc., have been found to be differentially regulated in animal models of PD (Li et al., 2008; Patel et al., 2007).

Cypermethrin induces the nigrostriatal dopaminergic neurodegeneration in adult rats and postnatal pre-exposure enhances the susceptibility, when re-challenged during adulthood (Singh et al., 2010). Cypermethrin-induced nigrostriatal neurodegeneration does not offer only additional evidence to environmental theory of PD but could also be a more relevant model system to understand the elusive aspects of sporadic PD, as it induces neurodegeneration after prolonged exposure, even more than that of maneb and paraquat co-exposures (Patel et al., 2006; Singh et al., 2010; Thiruchelvam et al., 2002; Tiwari et al., 2010). Although proteomic analyses of the nigrostriatal tissues have been performed for various PD models, it is inevitable and worthwhile to decipher the effect of cypermethrin on the nigrostriatal proteome profile owing to its uniqueness, importance and environmental relevance. Microglial activation plays a critical role in the maneb and paraquat-induced nigrostriatal dopaminergic neurodegeneration (Cicchetti et al., 2005; Saint-Pierre et al., 2006). Minocycline is a broad spectrum, anti-inflammatory and lipid soluble tetracycline antibiotic. Owing to its lipophilic nature, it easily enters the brain and inhibits microglial activation and produces anti-inflammatory effects thereby encounters nigrostriatal dopaminergic neuronal degeneration (He et al., 2004). For assessing the
contribution of microglial activation in the differential expression of nigrostriatal proteins and their subsequent contribution in neurodegeneration, the experiments were performed in the presence of minocycline, as it is one of the most widely used microglial activation inhibitors in the animal models of PD (He et al., 2004). Understanding the role played by microglial cells in cypermethrin-induced nigrostriatal dopaminergic neurodegeneration may provide novel information about its elusive aetiology. Aetiological insights of cypermethrin-induced nigrostriatal dopaminergic neurodegeneration could be of worth for designing the preventive and therapeutic strategies to encounter PD. The present study aimed to investigate the protein expression patterns in the substantia nigra and striatum of cypermethrin-exposed adult rats, which were also exposed to a non-toxic dose of cypermethrin during postnatal days 5-19, to identify the differentially expressed proteins and to examine the role of microglial cells therein, leading to motor impairment and onset of PD phenotype.

Materials and methods

Materials

Acrylamide, ammonium persulphate, anti-synaptosomal associated protein-25 (anti-SNAP-25) primary antibody, bovine serum albumin (BSA), bromophenol blue, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), cypermethrin, 3,3’-diaminobenzidine liquid enhanced system (DAB), 3,4-dihydroxybenzylamine hydrobromide (DHBA), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), 3-hydroxytyramine hydrochloride (dopamine), N,N’ methylene bisacrylamide, minocycline, nonidet P-40, sodium orthovanadate, paraformaldehyde, protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), polyvinylidene fluoride (PVDF) membrane, sodium dodecyl sulphate (SDS), sodium deoxycholate, tris-base, N,N,N’,N’-tetramethylethylenediamine (TEMED), tween-20 and urea
were purchased from Sigma-Aldrich, St. Louis, MO, USA. Immobiline pH gradient strips, immobiline pH gradient buffer and dry strip cover fluid were purchased from GE Healthcare, Chalfont, St.Giles, UK. Formaldehyde, glycerol, potassium dihydrogen orthophosphate, methanol, silver nitrate and sodium carbonate were procured from Merck Limited, Mumbai, India. Acetic acid, dibutyl phthalate xylene (DPX), sodium chloride, magnesium chloride, thiourea and xylene were procured from Sisco Research Laboratory, Mumbai, India. Agarose, alkaline phosphatase chromogen containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) western blot kit were purchased from Bangalore Genei, Bangalore, India. Anti-stathmin and anti-integrin-αM primary antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Frozen section medium Neg-50 was purchased from Richard Allen Scientific, Kalamazoo, MI, USA. Perchloric acid was purchased from Ranbaxy Private Limited, New Delhi, India.

**Animal treatment**

Animals (male Wistar rats) were obtained from the animal colony of the Indian Institute of Toxicology Research (IITR), Lucknow and kept in the animal house under the standard conditions (temperature- 22±2 centigrade ([degree]C), humidity- 45–55%, light: dark cycle- 12 hours (h); 12h and light intensity 300–400 lux) (Singh et al., 2010; Tiwari et al., 2010). During postnatal periods, pups were kept on mother milk as feed. Postnatal animals were divided into cypermethrin and cypermethrin plus minocycline treated groups along with the respective controls. Male pups were treated with cypermethrin [1.5 milligram (mg)/kilogram (kg)] intraperitoneally (ip), twice a week, during postnatal days 5-19. Control animals were injected with an equal volume of corn oil. After the postnatal treatments, animals were kept in normal conditions and re-challenged with cypermethrin (15 mg/kg; ip, twice a week) with or without
minocycline (30 mg/kg body weight, ip, daily) for 4, 8 and 12 weeks upon adulthood (Singh et al., 2010; Tiwari et al., 2010). No mortality was seen among the animals at the doses of cypermethrin and minocycline used in this study. The dose of cypermethrin was selected on the basis of previous study (Singh et al., 2010), in which the dose response of cypermethrin was checked. Adult animals were fed standard pellet diet and water ad libitum. Animals were sacrificed via the cervical dislocation; the brain was dissected out and kept immediately in liquid nitrogen. Dopamine content was measured on the same day; however, other experiments were performed within a week. The institutional ethics committee for the use of laboratory animals approved the study and the experiments comply with the current laws of animal experimentations in India.

Proteome profiling of the striatum and substantia nigra

The striatum and substantia nigra of controls and treated animals were dissected out and protein samples were prepared (Patel et al., 2007; Sinha et al., 2009). The substantia nigra or striatum dissected from two animals were pooled for proteomics experiments, as protein sample obtained from the tissue of one animal was not sufficient for proteome analysis particularly for coommassie brilliant blue staining. Secondly, the pooling of samples for proteomics minimizes the inter-animal variations. A total of 3 independent experiments with pooled samples were performed. The substantia nigra and striatum were separately homogenized in the lysis buffer. The homogenized content was sonicated and centrifuged at 10,000 xg for 20 minutes (min) at 4 °C. The supernatant was taken and used for further experiments. Protein content was measured and 2-D PAGE was carried out employing previously reported protocols (Patel et al., 2007; Sinha et al., 2009). The resultant gels were stained with brilliant blue R-250/silver nitrate. Both staining strategies were initially used to check the reproducibility in the expression level of
spots and also to avoid experimental variations arising due to staining. Only silver stained gels were used for analyzing the differential expression pattern of the proteins. Coomassie brilliant blue staining was used to check the presence of differentially expressed spots that were seen in the silver stained gels to rule out the possibility of false positive spots. The images of 2-D gels were compared using Image Master 2D platinum software. The size and colour intensity of gels were normalized and the spot volume/intensity calibration, spot detection, background subtraction and matching, etc., were performed with the software. In the 2-D gels, one spot, whose level of expression and location across all gels were unchanged, was used as landmark spot for analyses. Spot sizes/volumes of all the spots in individual gels were analyzed by Image Master 2D platinum software. The values of all the spots from independent sets of experiments were taken. These values of the differentially expressed spots (considered level: 15% changes in at least one treatment condition) were analyzed by ANOVA along with Bonferroni post-test. Equal loading and similar staining procedures were used for the gels considered for the study to minimize the biological variations arising due to sample loading and run-to-run variability (Patel et al., 2007; Sinha et al., 2009). The second dimensional gels used for this study were larger in size (gel casting assembly size: 24x18 centimeter (cm) in diameter) and the spots were properly resolved, therefore, the differentially expressed spots were picked manually.

Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) and liquid chromatography mass spectrometry (LC-MS) analyses

The silver stained gels were picked and used for protein extraction and MALDI-TOF/LC-MS analyses. The protein spots were cut from the gels, trypsinized and the digested peptides were dissolved in α-cyano-4-hydroxy cinnamic acid. MALDI-TOF and LC-MS analyses of the samples were performed and protein identity was established, as described elsewhere (Patel et
In brief, MALDI-TOF/TOF and 2-D Nano LC-electrospray ionization trap were used for mass spectrometric identification. Peaks of auto-digested trypsin were used as an internal standard to ensure mass accuracy. The MASCOT search engine based on National Centre for Biotechnology Information (NCBI) and Swiss Prot protein databases was used to identify peptide mass fingerprints. Bio-tools version 2.2, flex control and flex analysis softwares were used for acquisition and analyses. Peptide mass tolerance ±2 Da was the allowed error for matching the peptide values. Probability based MOWSE score was estimated as ion scores -10* log₁₀ (probability). Identification of proteins was confirmed with their molecular weights and isoelectric points in the gel (Patel et al., 2007; Sinha et al., 2009).

**Western blot analyses**

Western blot analyses, using standard procedure (Tiwari et al., 2010), validated the expression patterns of two differentially expressed proteins. Minimum 3 independent sets of experiments for all treatment groups were performed using one animal per group in each set. The expression level of β-actin, a house keeping protein was checked in the western blotting along with both the examined proteins. In brief, the proteins were electroblotted onto PVDF membrane after SDS-PAGE and incubated with anti-stathmin immunoglobulin G (rabbit polyclonal primary antibody; 1:500 dilution) or anti-SNAP-25 immunoglobulin G (rabbit monoclonal primary antibody) in tris buffered saline (TBS; pH 7.4)) containing 5% non-fat dry milk, overnight at 4 [degree]C. The blot was washed with TBS containing 0.2% tween-20 to remove the unbound antibodies and incubated further with anti-rabbit immunoglobulin G alkaline phosphatase conjugate developed in rabbit (1:5000 dilutions). The blot was extensively washed with TBS for 1h with 3-4 changes and developed with BCIP/NBT alkaline phosphatase substrate solution. Images were captured and the band density was calculated by computerized densitometry system (Alpha Imager...
System, Alpha Innotech Corporation, San Leandro, CA, U.S.A.). Band densities were normalized to β-actin.

**Microglial activation**

The coronal sections [20 micrometer ([μ]m) thick] were cut (Singh *et al.*, 2010) and microglial activation was performed, as described (Saint-Pierre *et al.*, 2006). In brief, the brains were perfused and kept serially in 10% paraformaldehyde and graded sucrose solution and endoperoxidase activity was minimized. Every third section was selected and the cells were counted within a defined frame bilaterally in 6 sections per animal. The values were averaged for each animal and 3 animals were used for each treatment group for analyses. The sections were incubated with monoclonal anti-integrin-αM rabbit polyclonal primary antibody (1:500) at 4 [degree]C for 48 h. After three washings of 15 min each with phosphate buffered saline, the sections were incubated with secondary antibody for 1 h and then with streptavidin-peroxidase complex for 30 min. The colour was developed with DAB substrate solution. The sections were dehydrated in graded ethanol and permanently mounted with DPX. The sections were visualized under the microscope at 20X magnification.

**Neuronal nuclei/tyrosine hydroxylase (NeuN/TH)-immunoreactivity**

Coronal sectioning, TH-immunoreactivity (TH-IR) and the counting of TH-immunoreactive cells and NeuN positive cells were performed (Singh *et al.*, 2010). Similarly, TH-immunoreactivity was also performed in the striatum. In brief; endogenous peroxidase activity and non-specific labeling were blocked. Every third section was selected and the cells were counted bilaterally in 6 sections per animal. The values were averaged for one animal and 3 animals were used for each treatment group for analyses. The sections were incubated with monoclonal anti-NeuN and anti-TH antibodies (only TH-antibody for the striatum) and further incubated with secondary
antibody, followed by streptavidin peroxidase complex. The colour was developed with DAB; the sections were dehydrated in graded ethanol and permanently mounted with DPX. The mounted sections were visualized under light microscope. Images were captured with charged coupled device (CCD) camera and unbiased counting of TH-positive cells in the substantia nigra was performed at 20X magnification (Leica Microscope DM6000 B, Germany) using a computerized image analysis system (QWin Pro, Leica, Germany) (Singh et al., 2010). The average survival is expressed as the percentage of TH-positive neurons in controls. Integrated density of TH-positive fibres in the striatum was calculated using freely available software ImageJ basic (version 1.38).

**Measurement of the striatal dopamine and neurobehavioral indices**

Dopamine was quantified in the striatum and the neurobehavioral indices were measured using rotarod and optovarimax (Singh et al., 2010). In brief, the striatum (10% w/v) was homogenized and the homogenate was centrifuged. Minimum 3 independent sets of experiments for all treatment groups were performed using one animal per group in each set. The dopamine content was measured in the filtrate using reverse phase C-18 column attached with high performance liquid chromatography (HPLC) coupled with electrochemical detector (Waters, Milford, MA, USA). The concentration of dopamine is expressed as nanogram (ng)/mg tissue. Similarly, the time of stay at rotarod and spontaneous locomotor activity in infrared beam-activated movement monitoring chamber were recorded. Initially, the animals were trained for 5 min per day for three days at rotarod at a speed of 5 revolutions per minute. The time spent on rod was recorded and the maximal observation time considered for this study was 5 min. The animals were kept in movement monitoring chamber for a min before taking the initial reading and spontaneous locomotor activity was recorded further for 5 min. A minimum of four experimental readings
was recorded for each animal and the results were averaged to obtain a single value. The experiments were performed with 5 animals (5 observations) for each treatment group and the average was calculated. A minimum of three sets of similar experiments (3 experiments x 5 observations) was performed for obtaining the final values.

**Statistical analysis**

Two-way analysis of variance (ANOVA) along with Bonferroni post-test was used for comparisons. The data are expressed as means ± standard error (SE) and ‘p’ value less than 0.05 was considered as significant.

**Results**

Cell bodies of dopaminergic neurons are localized in the substantia nigra and axons are projected into the striatum. As dopaminergic neurodegeneration leads to reduced level of dopamine in the striatum, which is mainly responsible for the regulation of the motor activities, therefore, the degenerative changes in the substantia nigra and striatum cannot be considered as totally independent events. The substantia nigra and striatum were analyzed independently in this study to identify the relationship between the differentially expressed proteins and neurodegenerative changes in both the regions of the brain as nigral protein could possibly regulate the degeneration of the cell body while the striatal proteins may play critical roles in the degeneration of axons or regulation of motor activities.

**Protein expression patterns and mass spectrometric analyses**

The differential expression (more than 10%) was observed in 36 protein spots at some or the other time of the treatment, identification of only those protein spots were performed, which could be unambiguously visualized even in coommassie brilliant blue staining, exhibited the differential expression of more than 15% and were statistically significant at least in one
treatment condition. MALDI-TOF and LC/MS identified 15 protein spots of 2-D gels (9 of the substantia nigra and 6 of the striatum) as ATP-SD chain, heat shock protein (Hsp)-70, truncated connexin-47, NADH2 dehydrogenase (ubiquinone) 24k chain precursor, α-internexin intermediate filament (α-IIF), mitogen activated protein kinase (MAPK) activated kinase-5, platelet-activating factor acetyl hydrolase, isoform 1b alpha 2 subunit (PAF-AH1b-α2), SNAP-25 and Hsp-60 in the substantia nigra and protein disulfide isomerise-ER 60 protease (PDI-ER 60 protease), NAD specific isocitrate dehydrogenase α-subunit (NAD-IDH α-subunit), phosphatidylethanolamine-binding protein 1 (PEBP-1), prohibitin, stathmin and ubiquitin conjugating enzyme in the striatum (Fig. 1, Fig. 2A and B). The PDI-ER 60 protease expression was attenuated after 8 and 12 weeks treated groups. The decrease was more pronounced in 12 weeks treated animals. The level of stathmin decreased in cypermethrin treated rats, which was restored in minocycline co-treated animals. NAD-IDH α-subunit expression was attenuated in cypermethrin treated groups. PEBP-1 expression was increased and prohibitin was decreased in 4 weeks treated animals; however, the expression of ubiquitin conjugating enzyme was decreased in 8 weeks treated groups. MAPK activated kinase-5 expression was increased while the expression of NADH2 dehydrogenase (ubiquinone) 24k chain precursor was decreased in 4 weeks treated animals. Hsp-70, PAF-AH 1b-α2, ATP-SD chain and SNAP-25 expressions were down regulated in cypermethrin treated animals. ATP-SD chain, PAF-AH1b-α2 and SNAP-25 levels were significantly restored with minocycline co-exposure in cypermethrin treated rats. An increased expression of α-IIF and Hsp60 were observed in cypermethrin treated rats, which was restored up to normal level in minocycline co-treated animals. Truncated connexin-47 expression was increased in 12 weeks cypermethrin-treated animals as compared with respective control. Truncated connexin-47 levels were restored in minocycline co-treated animals (Fig. 2A).
Minocycline alone did not produce any significant change in the treated animals as compared with controls (data not shown).

**Western blots analyses of SNAP-25 and stathmin**

SNAP-25 and stathmin, two differentially expressed proteins in 2-D PAGE, were randomly selected to validate the expression patterns of proteins employing western blot assays. Attenuation of SNAP-25 expression in the substantia nigra was time of exposure dependent, as obtained in 2-D PAGE. The expression of stathmin, involved in neuronal growth factor (NGF)-induced MAPK signaling and playing a key role in the differentiation of developing neurons, was reduced in the cypermethrin-treated rat striatum in a time of exposure dependent manner. The trends of the expression of these proteins in the presence or absence of minocycline were also similar (Fig. 3A-D), as observed in 2-D PAGE. No statistically significant change was observed either in minocycline alone (data not shown) or in cypermethrin and minocycline cotreated animals as compared with controls.

**Microglial activation**

Cypermethrin treatment increased the number of integrin-αM positive cells as measured by integrin-αM labelling. Increased microglial activation, i.e., an increased integrin-αM labelling, was observed in the substantia nigra of 4, 8 and 12 weeks cypermethrin treated adult rats, which were pre-exposed to cypermethrin during the postnatal days 5-19, as compared with respective controls. The increase in the number integrin-αM positive microglial cells was dependent on the time of cypermethrin exposure during adulthood. Animals treated with cypermethrin for 12 weeks during adulthood exhibited more pronounced increased as compared with 8 weeks treated animals and 8 weeks adulthood cypermethrin treated animals showed more integrin-αM positive cells in the substantia nigra as compared with 4 weeks adulthood cypermethrin treated animals.
Minocycline co-treatment restored the number of integrin-αM positive cells (activated microglia) in 4, 8 and 12 weeks adulthood cypermethrin treated rats (Fig. 4A-B). No statistically significant change was visualized either in minocycline alone (data not shown) or in cypermethrin and minocycline co-treated animals in comparison with controls.

Similarly, the number of integrin-αM positive cells was increased in the striatum of cypermethrin treated rats and minocycline co-exposure restored the number of microglial cells (Fig. 4 C-D).

**Neuronal nuclei (NeuN)/TH-immunoreactivity**

TH-IR and number of NeuN/TH immunoreactive cells were reduced in the substantia nigra of 4, 8 and 12 weeks cypermethrin treated adult rats, which were pre-exposed to cypermethrin during the postnatal days 5-19 as compared with respective controls. Minocycline co-treatment significantly restored the TH-IR and number of NeuN/TH immunoreactive cells in 4, 8 and 12 weeks cypermethrin treated adult rats, which were pre-exposed to cypermethrin during the postnatal days 5-19 (Fig. 5A-B). No statistically significant change was found either in minocycline alone (data not shown) or in cypermethrin and minocycline co-treated animals with respect to controls.

The fiber density of TH-positive neurons was reduced in the striatum of cypermethrin treated rats and the loss of neuronal fibers was restored by minocycline co-exposure in cypermethrin and minocycline co-treated rats in a time of exposure dependent manner (Fig. 5 C-D).

**Striatal dopamine level**

Dopamine content was reduced in the substantia nigra of 4, 8 and 12 weeks cypermethrin treated adult rats, which were pre-exposed to cypermethrin during the postnatal days 5-19, as compared with respective controls. Minocycline co-treatment significantly restored the dopamine content
in the striatum of 4, 8 and 12 weeks cypermethrin treated adult rats, which were pre-exposed to cypermethrin during the postnatal days 5-19 (Fig. 6A). Minocycline alone treated animals did not exhibit any statistically significant change in dopamine level as compared with controls (data not shown).

**Behavioural studies**

Exposure to cypermethrin produced significant impairment in motor activities (Fig. 6B-C). The time spent on rotarod and distances traveled by the animals during spontaneous locomotor activity were reduced in cypermethrin treated rats as compared with controls. Minocycline co-treatment significantly recovered neurobehavioral indices of cypermethrin treated rats (Fig. 6B-C). Minocycline alone treated animals did not reflect any statistically significant change as compared with controls (data not shown).

**Statistical analysis**

The statistical analyses, which include, p-values, t-values, F-values and df-values, of all the figures are summarized in Table 1.

**Discussion**

Owing to quick metabolism and elimination from the body, cypermethrin, in general, does not accumulate in the environment and is not severely toxic (Bradberry *et al.*, 2005). However, the widespread and indiscriminate usages of cypermethrin raise concerns about its non-specific effects on the environment and on the non-target organisms, including humans (Bradberry *et al.*, 2005). Cypermethrin is a well established modulator of gamma-aminobutyric acid and dopamine levels in brain (Singh *et al.*, 2010). It is a well known fact that exposure to pesticides, including cypermethrin, determines progressive damage of the dopaminergic neurons in the substantia nigra (Logroscino, 2005; Tiwari *et al.*, 2010; Singh *et al.*, 2010). Cypermethrin induces the
nigrostriatal dopaminergic neurodegeneration either alone or in combination with other neurotoxicants (Singh et al., 2010; Tiwari et al., 2010; Giray et al., 2001; Kale et al., 1999).

For deciphering the link between differential expression patterns of proteins and microglial activation in the striatum and substantia nigra of cypermethrin-treated animals, effects of minocycline therein and their subsequent contribution to cypermethrin-mediated nigrostriatal dopaminergic neurodegeneration, the effect of cypermethrin on the indices of the nigrostriatal dopaminergic neurodegeneration (such as, TH-immunoreactivity) was measured in the present study as described previously (Singh et al., 2010) along with minocycline treated animals and respective controls. Cypermethrin treated adult rats, which were also treated during the postnatal days 5-19, were used in this study, as this treatment paradigm is found to produce maximum effects (Singh et al., 2010). An increased oxidative stress and decreased expression of anti-apoptotic proteins play critical roles in neuronal apoptosis (Harbour and Dean, 2000). An increased expression of PEBP-1 at early time point could act as a regulator to initiate apoptosis and neurodegeneration by regulating cell survival pathways. PEBP-1 regulates growth and differentiation at spindle checkpoint through protein kinase C (PKC), an important mediator in the signal transduction events, and inhibits MAP kinase signalling (Eves et al., 2006). It inhibits nuclear factor-κB (NF-κB) signaling, which is required for cell survival (Eves et al., 2006). This is further supported by a significant decrease in the expression of prohibitin, which possesses transcriptional regulatory and p53-mediated anti-apoptotic activities. The increased PEBP-1 and decreased prohibitin could contribute to neuronal damage, as down regulation of the latter activates pro-apoptotic machinery. Decreased SNAP-25 expression in cypermethrin treated rats and significant recovery in minocycline co-treated animals were observed in the substantia nigra, which could be associated with the degeneration of dopaminergic neurons, as SNAP-25 acts as a
pre-synaptic plasma membrane protein and regulates synaptic vesicle fusion and neurotransmitter release (Hodel, 1998). A time dependent decrease in the expression of stathmin, a potent inhibitor of microtubule assembly and a major constituent of the neuronal cytoskeleton, and significant recovery in its expression in minocycline co-treated animals indicated towards the role of stathmin in altered structural integrity and degeneration of neurons, as it contributes to cellular integrity (Giampirtro et al., 2005; Jin et al., 2004). This is further supported by an augmented expression of α-IIF, which contributes to neurotoxicity owing to its abnormal neurofilaments accumulation property (Cairns et al., 2004; Ching et al., 1999). The results obtained are in accordance with the previous observations, which correlate perturbations in stathmin and α-IIF expressions with neurological disorders (Cairns et al., 2004; Ching et al., 1999). An alteration in PAF-AH 1b-α2 expression could possibly be associated with neuronal degeneration. PAF-AH 1b-α2 is a non-catalytic subunit of an acetyl hydrolase complex that inactivates platelet-activating factor by removing the acetyl group, which is required for actin polymerisation and plays an important role in brain development, cell proliferation and neuronal migration (Tsai et al., 2005).

Hsp is a class of molecular chaperones and its expression alters in response to oxidative stress and the accumulation of misfolded proteins. As Hsp-60 is a mitochondrial protein and is involved in the formation of a complex required for protein folding and normal functioning of mitochondria, decrease in its expression indicated the possible role of mitochondrial dysfunction and energy metabolism in cypermethrin model system as reported in PD. Similarly, Hsp-70 is involved in protein translocation across mitochondrial membranes and in the delivery of misfolded proteins to proteolytic enzymes in the mitochondrial matrix; therefore, up-regulation in its expression could act as an adaptive mechanism to encounter the nigrostriatal dopaminergic
neurodegeneration (Dong et al., 2006). Hsp-70 suppresses the toxicity induced by misfolded proteins in PD (Witt, 2010). Significant decrease in its expression in cypermethrin treated rats could assist the aggravating toxicity. Increased expression of Hsp-60 could be due to bioaccumulation of misfolded protein during stress (DiDomenico et al., 2010). Decreased expression of ubiquitin conjugating enzyme could be associated with the onset of neurotoxicity leading to PD phenotype, as it produces adverse effect on the cell proteasomal machinery (Obin et al., 1998). The decreased level of PDI-ER 60 protease, a component of the proteolytic machinery involved in the degradation of misfolded proteins could be related to PD pathogenesis, as it contributes to the accumulation of misfolded proteins (Otsu et al., 1995). An altered energy metabolism in PD phenotype is extensively reported and cypermethrin reduced the expression of NAD-IDH, NADH2 dehydrogenase (ubiquinone) 24k chain precursor and ATP-SD chain, which are involved in ATP production (Ying et al., 2007). Energy deprivation may also be responsible for neuronal damage in cypermethrin treated rats in this study. The similar protein expression patterns of stathmin, ATP-SD chain, mitochondrial NADH dehydrogenase (ubiquinone), etc., showed the resemblance of cypermethrin-induced neurodegeneration with other model systems. However, lack of similar expression patterns of a few reported proteins and unique pattern of some novel proteins have shown the uniqueness of this model system (Basso et al., 2004; Li et al., 2008; Patel et al., 2007; Werner et al., 2008).

The decreased levels of dopamine, TH-IR positive cells and impaired behavioural indices have been associated with cypermethrin-induced nigrostriatal dopaminergic neurodegeneration (Singh et al., 2010; Tiwari et al., 2010). Minocycline, a microglial activation inhibitor, significantly restored the level of dopamine, TH-IR positive cells and behavioural indices showing that microglial activation is an important and critical event in the regulation of cypermethrin-induced
nigrostriatal dopaminergic neurodegeneration. This is in accordance with the previous reports, which have shown that microglial activation is a critical event in pesticides-induced neurodegeneration (Cicchetti et al., 2005; Saint-Pierre et al, 2006). Minocycline co-treatment restored the expression of seven proteins i.e., stathmin, SNAP-25, PAF-AH1b-2α, α-IIIF, ATP-SD chain, truncated connexin-47 and Hsp-60 in the cypermethrin-induced nigrostriatal dopaminergic neurodegeneration. The study shows that the nigrostriatal dopaminergic neurodegeneration and the expression levels of seven proteins were significantly dependent on the microglial activation (Cicchetti et al., 2005; Saint-Pierre et al, 2006). Furthermore, an increased expression of truncated connexin-47 in 12 weeks cypermethrin treated animals could be due to an increased free radical generation leading to the hyper-activation of microglia and subsequent release of bioactive molecules, which increases the activity of hemi-channels, reduces communication between gap junctions and enhances apoptotic cell death (Nagya et al., 1996; Orellana et al., 2009). Proteome patterns of any of the studied model systems do not mimic those observed in sporadic PD or even in other model systems significantly owing to the fact that proteome patterns are highly dynamic and depend on the type and strain of animals, chemicals used to elicit the disease, fractions of the biological materials used, environmental conditions at the time of tissue isolation and disease pathogenesis and even the life style factors. This is the first study performed using cypermethrin model system employing proteomics approach; therefore, whole striatum or substantia nigra proteome analyses were performed rather than sub-cellular proteomics, such as mitochondrial proteomics. As this approach does not remove the highly abundant proteins; therefore, the differential expression of a few specific proteins could not be detected (Patel et al., 2008), however, all the proteins identified in this study play critical roles in neurological diseases, including PD (Cairns et al., 2004; Ching et al.,
The expression levels of eight proteins were not significantly altered by microglial activation inhibitor showing that their expressions could possibly be regulated by intermediary events leading to microglial activation or microglial activation independent events, as that proteins regulate mitochondrial dysfunction, energy production, free radical generation, synaptic transmission and/or apoptosis (Patel et al., 2008; Singh et al., 2010). This study demonstrates that microglial activation dependent and independent pathways in combination lead to cypermethrin-induced nigrostriatal dopaminergic neurodegeneration.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


Legend to figures

FIG. 1. 2-D gel electrophoretograms of the substantia nigra (A) and striatum (B) of control and cypermethrin treated rats. The location of differentially expressed proteins in the gels along with their identity, which were established following mass spectrometry of the spots and database search for homology, are also shown.

FIG. 2. Bar diagrams representing differentially expressed proteins in the substantia nigra and striatum of control and cypermethrin treated rats with or without minocycline co-exposure in terms of spot volumes, expressed as percent of control, are shown in panels A and B respectively (n=3).

FIG. 3. Western blots of SNAP-25 in the substantia nigra and stathmin in the striatum of controls and cypermethrin treated rats with or without minocycline co-exposure. The protein expression pattern of SNAP-25 in the substantia nigra is shown in panel A and the corresponding band density ratio with respect to the constitutively expressed protein β- actin is plotted in the panel B. Similarly, the protein expression pattern of stathmin in the striatum and the corresponding band density ratio with respect to β- actin is shown in panels C and D respectively. Lanes 1, 5 and 9 represent controls; 2, 6 and 10 represent minocycline alone treated; 3, 7 and 11 represent cypermethrin treated and 4, 8, 12 represent minocycline and cypermethrin co-treated groups (n=3).

FIG. 4. Effect of cypermethrin on microglial activation in the substantia nigra and striatum in the presence or absence of minocycline co-treatment along with respective controls. A, D and G represent control B, E and H represent cypermethrin treated and C, F and I represent cypermethrin and minocycline co-treated rats’ substantia nigra (A). Bar diagram showing the number of integrin αM positive cells in the substantia nigra of control and treated animals (B).
A, D and G represent control B, E and H represent cypermethrin treated and C, F and I represent cypermethrin and minocycline co-treated rats’ striatum (C). Bar diagram showing the number of integrin αM positive cells in the striatum of control and treated animals (D) (n=3).

**FIG. 5.** Effect of cypermethrin on NeuN/TH-immunoreactivity in the substantia nigra and TH-immunoreactivity in the striatum with or without minocycline along with respective controls. A, D and G represent control, B, E and H represent cypermethrin treated and C, F and I represent cypermethrin and minocycline co-treated rats’ substantia nigra (A). Bar diagram showing the number of TH/NeuN-positive cells in the substantia nigra (B). A, D and G represent control, B, E and H represent cypermethrin treated and C, F and I represent cypermethrin and minocycline co-treated rats’ striatum (C). Bar diagram showing the integrated density of TH-positive fibres in the striatum of control and treated animals (D) (n=3).

**FIG. 6.** Bar diagrams showing the striatal dopamine content (A), time of stay of experimental animals on rotarod (B) and distance traveled in the chamber (C) in the cypermethrin-treated rats with or without minocycline treatment along with respective controls (n=3 for dopamine and 3 sets of independent experiments using 5 animals per set for other variables).
TABLE 1: Summary of the p-values, t-values, F-values (interactions, treatment groups and time of exposures) and df-values (interactions, treatment groups, time of exposures and residual values) obtained after statistical analyses.

<table>
<thead>
<tr>
<th>FIG.</th>
<th>p-Value/ t-Value</th>
<th>F-Value</th>
<th>df-Value</th>
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<tr>
<td>2. (A)</td>
<td>The p-values are denoted as * (p&lt;0.05) versus (vs.) control and γ (p&lt;0.05) vs. cypermethrin treated rats and the t values respectively at 4; 8; 12 weeks are t=3.10; 2.95; 2.90 vs. control and t=2.74; 3.42; 3.52 vs. cypermethrin treated rats (α-IIF)</td>
<td>0.048; 15.4; 0.009</td>
<td>4, 2, 2, 18</td>
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<td>The p-value is denoted as ** (p&lt;0.01) vs. control and the t value at 4 weeks is t=4.18 vs. control (NADH2 dehydrogenase 24k chain precursor)</td>
<td>2.06; 10.48; 7.88</td>
<td>4, 2, 2, 18</td>
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<td>The p-values are denoted as ** (p&lt;0.01) vs. control and γ (p&lt;0.05) vs. cypermethrin treated rats and the t value at 12 weeks is t=4.30 vs. control and t=3.15 vs. cypermethrin treated rats (truncated connexin-47)</td>
<td>0.844; 13.8; 2.4</td>
<td>4, 2, 2, 18</td>
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<td>The p-values are denoted as * (p&lt;0.05) and ** (p&lt;0.01) vs. control and the t values at 4; 8 weeks are t=3.45; 2.88 and t=2.89 at 4weeks respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (Hsp-70)</td>
<td>0.96; 9.21; 2.91</td>
<td>4, 2, 2, 18</td>
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<td>Description</td>
<td>p-values</td>
<td>t-values</td>
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<td>The p-values are denoted as * (p&lt;0.05) and ** (p&lt;0.01) vs. control and γ (p&lt;0.05), γγ (p&lt;0.01) vs. cypermethrin treated rats and the t values respectively at 4; 8; 12 weeks are t=4.10; 3.10; 3.60 vs. control and t=4.28; 3.08; 3.42 vs. cypermethrin treated rats (PAF-AH 1b-α2)</td>
<td>1.89; 7.37; 3.72</td>
<td>4, 2, 2, 18</td>
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<td>The p-values are denoted as * (p&lt;0.05) vs. control and γ (p&lt;0.05) vs. cypermethrin treated rats and the t values respectively at 4; 8 weeks are t=3.29; 3.28 vs. control and t=3.42 at 4 weeks vs. cypermethrin treated rats (Hsp-60)</td>
<td>0.13; 17.38; 0.44</td>
<td>4, 2, 2, 18</td>
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<td>The p-values are denoted as * (p&lt;0.05) and ** (p&lt;0.01) vs. control and the t values respectively at 4 weeks are t=4.67; 3.31 respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (MAPK activated kinase-5)</td>
<td>2.54; 8.71; 7.85</td>
<td>4, 2, 2, 18</td>
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<td>The p-values are denoted as ** (p&lt;0.01) and *** (p&lt;0.001) vs. control and γ (p&lt;0.05), γγ (p&lt;0.01) vs. cypermethrin treated rats and the t values respectively at 4; 8; 12 weeks are t=4.00; 4.42; 6.33 vs. control and t=3.03; 2.79; 3.59 vs. cypermethrin treated rats (ATP-SD chain)</td>
<td>0.82; 37.43; 2.96</td>
<td>4, 2, 2, 18</td>
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<td>The p-values are denoted as * (p&lt;0.05) and ** (p&lt;0.01) vs. control and γ (p&lt;0.05) vs. cypermethrin treated rats and the t values respectively at 8; 12 weeks are t=3.35; 4.08 vs. control and t=3.27 at 12 weeks vs. cypermethrin treated rats (SNAP-25)</td>
<td>0.97; 10.82; 1.65</td>
<td>4, 2, 18</td>
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<td>The p-values are denoted as * (p&lt;0.05), ** (p&lt;0.01) and *** (p&lt;0.001) vs. control and the t values at 8; 12 weeks are t=3.52; 4.85 and t=3.09; 4.27 respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (PDI-ER 60 protease)</td>
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<td>The p-values are denoted as *** (p&lt;0.001) vs. control and γγ (p&lt;0.01) vs. cypermethrin treated rats and the t values respectively at 8; 12 weeks are t=4.48; 5.72 vs. control and t=3.49; 3.94 vs. cypermethrin treated rats (stathmin)</td>
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<td>The p-value is denoted as ** (p&lt;0.01) vs. control and the t values at 4 weeks are t=3.77; 3.40 respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (prohibitin)</td>
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<td>The p-values are denoted as ** (p&lt;0.01) and *** (p&lt;0.001) vs. control and the t values at 4 weeks are t=4.42; 3.71 respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (PEBP-1)</td>
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<td>The p-values are denoted as * (p&lt;0.05), ** (p&lt;0.01) and *** (p&lt;0.001) vs. control and the t values at 4; 8; 12 weeks are t=4.03; 4.10; 5.10 and t=3.88; 3.37; 3.95 respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (NAD-IDH α-subunit)</td>
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<td>The p-values are denoted as * (p&lt;0.05) and ** (p&lt;0.01) vs. control and the t values at 8 weeks are t=3.64; 3.22 respectively in cypermethrin alone and cypermethrin and minocycline co-treated rats vs. control (ubiquitin conjugating</td>
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3. (B) The p-values are denoted as * (p<0.05), *** (p<0.001), γ (p<0.05), γγγ (p<0.001), # (p<0.05) and ### (p<0.001) and the t values are t= 3.51 at 4 weeks, t=5.45; 8.41 at 8 and 12 weeks, t=3.7 at 8 weeks, t=5.56 at 12 weeks, t=3.59 at 4 weeks and t=5.69; 8.27 at 8 and 12 weeks vs. control, vs. cypermethrin treated and vs. minocycline treated rats, respectively

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<td>2.9, 45.09, 6, 3, 2, 24</td>
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3. (D) The p-values are denoted as *** (p<0.001), γ (p<0.05), γγγ (p<0.001) and ### (p<0.001) and the t values are t=5.42; 8.24 at 8; 12 weeks, t=3.5 at 8 weeks, t=5.18 at 12 weeks and t=5.13; 8.06 at 8; 12 weeks vs. control, vs. cypermethrin treated and vs. minocycline treated rats, respectively

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<td>7.16, 27.88, 14.78, 6, 3, 2, 24</td>
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4. (B) The p-values are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001), γγγ (p<0.001) and the t values are t=2.80 at 4 weeks, t=4.02 at 8 weeks, t=10.33 at 12 weeks and t=7.95 at 12 weeks vs. control and vs. cypermethrin treated rats, respectively

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<td>9.31, 52.29, 16.00, 4, 2, 2, 18</td>
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4. (D) The p-values are denoted as ** (p<0.01), *** (p<0.001), γγγ (p<0.001) and the t values are t=3.98 at 8 weeks, t=9.06 at 12 weeks and t=6.02 at 12 weeks vs. control and vs. cypermethrin treated rats, respectively

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<td>5.5, 44.74, 13.45, 4, 2, 2, 18</td>
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5. (B) The p-values are denoted as ** (p<0.01), *** (p<0.001), γγ (p<0.01) and the t values are t=3.73 at 8 weeks, t=5.98 at 12 weeks and t=4.38 at 12 weeks vs. control and vs. cypermethrin treated rats, respectively

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<td>2.1, 24.21, 3.94, 4, 2, 2, 18</td>
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5. (D) The p-values are denoted as * (p<0.05), *** (p<0.001) and γ (p<0.05) and the t values are t=3.37 at 8 weeks, t=5.34 at 12 weeks and t=3.45 at 12 weeks vs. control and vs. cypermethrin treated rats, respectively

6. (A) The p-values are denoted as * (p<0.05), *** (p<0.001) and γγ (p<0.01) and the t values are t=2.78 at 4 weeks, t=4.43; 6.95, at 8, 12 weeks, t=4.15, at 12 weeks and t=2.79 at 12 weeks vs. control, vs. cypermethrin treated rats and vs. control in cypermethrin and minocycline co-treated rats, respectively

76. (B) The p-values are denoted as * (p<0.05), ** (p<0.01), *** (p<0.001) and γγ (p<0.01) and the t values are t=3.32; 4.29; 6.97 at 4, 8 and 12 weeks, t=4.29, at 12 weeks and t=2.68 at 12 weeks vs. control, vs. cypermethrin treated rats and vs. control in cypermethrin and minocycline co-treated rats, respectively

6. (C) The p-values are denoted as ** (p<0.01), *** (p<0.001), γ (p<0.05) and γγ (p<0.01) and the t values are t=3.71; 4.92; 7.17 at 4, 8 and 12 weeks, t=4.14 at 12 weeks and t=2.72 at 12 weeks vs. control, vs. cypermethrin treated rats and vs. control in cypermethrin and minocycline co-treated rats, respectively
[Image: Western blot showing the expression of Stathmin (19kDa) and β-actin over 4, 8, and 12 weeks]

54x13mm (300 x 300 DPI)
Toxicological Sciences

81x59mm (300 x 300 DPI)
70x49mm (300 x 300 DPI)
Control  |  Cypermethrin  |  Cypermethrin + minocycline

4 Weeks  

D  |  E  |  F

8 Weeks  

G  |  H  |  I

12 Weeks

70x49mm (300 x 300 DPI)
Diagram showing time on rotarod (% of control) over 4, 8, and 12 weeks for Control, Cypermethrin, and Cypermethrin + minocycline conditions.

60x31mm (300 x 300 DPI)