Neurotoxicology 74 (2019) 184–195

Contents lists available at ScienceDirect

Neurotoxicology

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

Full Length Article

A proteomic approach to investigate enhanced responsiveness in rechallenged adult rats prenatally exposed to lindane

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ARTICLE INFO

Keywords:
Lindane
Rat
Offspring
Rechallenge
Brain
Proteomic

ABSTRACT

Proteomic analysis was carried out in substantia nigra (SNi) and hippocampus (Hi) isolated from rat offspring born to mothers exposed to lindane (orally; 0.25 mg/kg) from gestation day 5 (GD5) to GD 21 and subsequently rechallenged (orally; 2.5 mg/kg X 21 days) at adulthood (12 weeks). 2D gel electrophoresis revealed no significant differences in the expression of proteins in brain regions isolated from prenatally exposed offspring at adulthood. Significantly greater magnitude of alterations was observed in the expression of proteins related to mitochondrial and energy metabolism, ubiquitin-proteasome pathway, structural and axonal growth leading to increased oxidative stress in Hi and SNi isolated from rechallenged offspring when compared to control offspring treated postnatally with lindane. Western blotting and DNA laddering showed a greater magnitude of increase in apoptosis in the Hi and SNi of rechallenged offspring. Ultrastructural analysis demonstrated disrupted mitochondrial integrity, synaptic disruption and necrotic structures in the brain region of rechallenged offspring. Neurobehavioral studies also demonstrated a greater magnitude of alterations in cognitive and motor functions in rechallenged rats. The data suggest that prenatal exposure of lindane induces persistent molecular changes in the nervous system of offspring which are unmasked leading to neurodegeneration following rechallenge at adulthood.

1. Introduction

Epidemiological studies have indicated that prenatal and childhood exposure of pesticide can influence the neurodevelopment in children (Eskenazi et al., 2006; Jurewicz and Hanke, 2008). There are evidences to suggest that exposure to organochlorine pesticides *including lindane* may have a role in the etiology of neurodegenerative diseases (Corrigan et al., 2000; Saravi and Dehpour, 2016). Even though the use and production of lindane, an organochlorine pesticide is banned in most of countries including India, lindane continues to be used in cosmetics, pharmaceutical and veterinary purposes. US Food and Drug Administration (FDA) has approved the use of lindane in the second-line treatment for scabies and pediculus (Goldust et al., 2013). Lindane is reported to persist in the environment due to its longer half life and slow degradation rate (Phillips et al., 2005; Vega et al., 2016). The concentrations of lindane in environment have been reported to be higher than the human exposure limit (Barron et al., 2017; Chakraborty et al., 2015; Pereira et al., 2010).

The residues of lindane have been reported in postmortem brain tissue and blood of patients suffering from Alzheimer's disease (AD) and Parkinson's disease (PD) (Corrigan et al., 2000; Fleming et al., 1994; Richardson et al., 2014). Experimental studies have also shown that

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Abbreviations: SNi, Substantia nigra; Hi, Hippocampus; AD, Alzheimer's disease; PD, Parkinson's disease; DS, Down syndrome; UPP, Ubiquitinproteasome pathway; GD, Gestation day; SLA, Spontaneous locomotor activity; SOD, Superoxide dismutase; PMSF, phenylmethylsulphonyl fluoride; DTT, Dithioerythritol; CCytC, Cytochrome

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https://doi.org/10.1016/j.neuro.2019.07.004
Received 23 January 2019; Received in revised form 17 July 2019; Accepted 18 July 2019

organochlorine pesticides induce neurodevelopmental effects in animals such as cerebral anomalies, autism, anxiety, memory loss etc. (Eriksson and Talts, 2000; Saravi and Dehpour, 2016). Blockade of chloride channels associated with GABA and glycine receptor are the primary cause of lindane induced neurotoxicity (Llorens et al., 1989; Vale et al., 2003). *in vitro* and *in vivo* studies have reported elevated oxidative stress as an important mechanism involved in lindane induced neurotoxicity (Arisi et al., 1994; Heusinkveld et al., 2010; Mladenović et al., 2010; Srivastava and Shivanandappa, 2005). Experimental studies have shown that lindane induces cell death via apoptosis necrosis and autophagy in brain as well as in the neuronal and non-neuronal cells under *in vitro* conditions by targeting protein associated with these cell death processes (Mudawal et al., 2015, 2018; Zucchini-Pascal et al., 2009).

Studies from our laboratory have shown that prenatal exposure of low doses of lindane leads to overexpression of cerebral and hepatic drug metabolizing enzymes, known to be involved in its metabolism and toxicity, in offspring during postnatal development (Johri et al., 2007). Rechallenge of prenatally exposed offspring led to a significant increase in the incidence of lindane induced convulsions in prenatally exposed offspring when compared to control offspring treated postnatally with lindane (Johri et al., 2008). Rechallenge of endosulfan, an organochlorine pesticide and/ or zineb, the fungicide, to adult mice, resulted in neurochemical changes which did not occur in mice exposed only during early development (Jia and Misra, 2007). Likewise, rechallenge with paraquat, a herbicide, and maneb, a fungicide to adult mice leads to the unmasking of silent toxicity resulting in enhanced adult susceptibility to these pesticides (Thiruchelvam et al., 2002). It has been suggested that early life exposure may induce persistent biochemical or behavioral changes in the nervous system that may not promote apparent signs of toxicity unless exposed to unmasking challenges (Barlow et al., 2007; Kraft et al., 2016).

Proteomic approaches have been used to identify the mechanism involved in neurodegenerative disorders and identify the molecular functions of proteins involved in neurodegeneration (Butterfield and Reed, 2016; Nayak et al., 2015; Shi et al., 2009). Studies in protein extract of substantia nigra (SNi) and hippocampus (Hi) isolated from postmortem tissue of patients of PD and AD revealed that development of these diseases is associated with significant alterations in expression of the proteins associated with the antioxidant system, mitochondrial function, energy metabolism and cytoskeletal dynamics. These proteomic studies also suggested a link between oxidative stress and altered protein levels in the diseased brain (Basso et al., 2004; Sultana et al., 2010). Proteomic changes after prenatal stress and hypoxia and hyperoxia in developing rat brain also revealed changes in proteins involved in oxidative stress, energy metabolism, synaptic functions, cytoskeletal dynamics and signal transduction suggesting an appearance of depressive phenotype at adult life and dysregulation of developmental processes due to oxidative stress (Kaindl et al., 2006; Mairesse et al., 2012). 2D and immunoblotting based proteomic studies from our laboratory have also demonstrated that elevated oxidative stress and depletion of antioxidant enzymes following low dose exposure of lindane in immature, adult and aged rats is associated with alterations in the expression of proteins involved in mitochondrial function, disrupted ubiquitin proteosome pathway (UPP) and molecular chaperons, calcium homeostasis, synaptogenesis, autophagy and apoptosis (Mudawal et al., 2015, 2018).

To further validate if low dose exposure of pesticide during a critical period of development has the potential to modify cellular function in offspring to make them more vulnerable, studies were initiated to evaluate the proteome-wide changes in Hi and SNi isolated from of rat offspring prenatally exposed to lindane (1/350th of LD50) and subsequently rechallenged at adulthood. Studies were also initiated to correlate the alteration in the expression of various classes of proteins with oxidative stress, apoptosis, neurobehavioral and ultrastructural changes in Hi and SNi isolated from prenatally exposed rats offspring rechallenged at adulthood.

2. Method and materials

2.1. Treatment of animals

Pregnant Wistar rats were obtained from the animal house facility of the CSIR-IITR, Lucknow and maintained according to the policy laid down by the Institutional Animal Care Committee. Rats were divided into two groups of 25 pregnant rats each. Rats in group 1 received 0.25 mg/kg b. wt., orally of lindane from gestation day 5 (GD5) to GD21. Untreated pregnant rats in group 2 served as controls and received equal amount of corn oil in an identical manner. This dose of lindane was based on our previous dose-dependent report on the prenatal effect of lindane on cerebral cytochrome P450 in rat offsprings (Johri et al., 2007). On the day of parturition, the average litter size was adjusted to eight per dam in all the groups with the equal number of males and females as far as possible to minimize any developmental effects on offspring. However, all the experiments were performed in males only. The male offspring born to the controls or lindane treated dams were divided into four groups of 50 offspring each at 12 weeks. In group I, the offspring born to control dams received corn oil orally for 21 consecutive days and served as the controls. In group II, the offspring born to lindane treated dams also received corn oil orally for 21 consecutive days and served as the prenatally exposed group. In group III, the offspring born to control dams received 2.5 mg/kg bw lindane, orally for 21 consecutive days while in group IV, the offspring born to lindane treated dams were exposed similarly to lindane (2.5 mg/kg, orally) and served as the rechallenged group. Offspring in different groups were sacrificed 24 h after the last dose by decapitation. After sacrifice, Hi and SNi were immediately dissected out from the brain and processed for isolation of tissue lysate for proteomic, DNA laddering/ fragmentation studies and biochemical analysis. The separate set of six rats in each group were monitored for neurobehavioral activity.

2.2. Neurobehavioral studies

2.2.1. Spontaneous locomotor activity (SLA)

Control and lindane treated rats were monitored for spontaneous locomotor activity (SLA) as described previously (Mudawal et al., 2018). Briefly, the instrument, Optovarimex- Apple II System consists a cage (45 cm × 45 cm) with two arrays of 15 infrared beams (distanced by 25.4 mm) perpendicular to each other that generates an electric impulse which upon each interruption is processed by the microcomputer. The basic parameters observed in the system were distance travelled, resting time, moving time, number of rearings and stereotypic counts. Distance travelled (in cm) is the measure of total distance travelled by the animal in the activity chamber whereas time spent (in seconds) while ambulatory activity denoted as moving time. Resting time denotes the total time spent by the animal in resting phase when no interruptions in any beam occur for at least 0.5 s. Number of rearings represent the number of interruptions caused to the photometer beam during vertical movement (standing), stereotypic count denotes the stereotypic activity of the animal. This system eliminates the bias in quantification of motility. Rat offspring were acclimatized for 15 min in experimental room and then placed individually in the activity monitor cage after which the above mentioned parameters were recorded for 5 min simultaneously. To prevent interference due to animal odors, the monitor cage was subsequently swabbed with 70% alcohol.

2.2.2. Continuous alternation test (Y-maze test)

Rat offspring's of control and lindane treated groups were assessed for spatial memory using a computerized Y-maze apparatus (TSE, Germany) as described earlier (Mudawal et al., 2018). Y-maze apparatus consisted of three identical arms of the dimensions, 40 cm length X 15 cm width X 30 cm height, with a conductive grid floor. The three arms were symmetrically oriented equilaterally with respect to each other. Rats were individually placed at the end of one arm, allowed to move freely for 5 min and tested for spatial memory alterations. Sequence of entry was automatically recorded on computer for each rat. Spatial memory refers to number and sequence of entries into the arms. The Y maze test readings have been calculated in terms of percentage alterations, measured as number of entries into 3 different arms divided by total number of possible entries, multiplied by 100 in control and lindane treated offsprings.

2.3. *Biochemical assays*

Hi and SNi isolated from six offspring each of control and lindane treated groups were processed separately for biochemical analysis. In brief, 10% homogenate was prepared in 0.1 M phosphate buffer pH 7.0. The tissue homogenates were then used for lipid peroxidation assay, reduced glutathione content (reduced GSH), superoxide dismutase (SOD) assay, catalase assay, protein carbonyl assay as described previously.(Boyland and Chasseaud, 1969; Dalle-Donne et al., 2003; Kakkar et al., 1984; Ohkawa et al., 1979; Sinha, 1972)

2.4. *Two-dimensional gel electrophoresis*

Hi isolated from three control or treated group and SNi isolated from six control or treated groups were pooled and generated three sets of pooled samples for studying brain regions in order to minimize inter-animal variations. To obtain whole protein fraction, tissues were homogenized in urea lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate), 2% triton X-100, 65 mM 1,4-dithioerythritol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), I mM EDTA 0.5% carrier ampholytes 3.5–10 and protease inhibitor on ice. Whole protein fraction was incubated on ice for one hr and then centrifuged at 10,000 × *g* at 4 °C for 30 min. The supernatant was then precipitated with methanol chloroform precipitation method as described previously (Fic et al., 2010). The protein pellet obtained was then resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM tris/HCl pH 9.0, 50 mMDTT, 0.2% Biolyte 3/10 ampholyte, 0.001% bromophenol blue). Protein content was quantified using 2D quant kit (GE healthcare UK). The reduction and alkylation of proteins were performed prior to IEF as described previously to achieve higher resolution (Liang et al., 2014).

Two- dimensional gel electrophoresis was carried out as reported previously (Fountoulakis, 2004). Briefly, isoelectric focusing was carried out using 11-cm immobilized pH gradient strips (IPG, 3–10 nonlinear pH gradients, Bio-Rad). Overnight in-gel rehydration of strips was performed in accordance with the manufacturer's recommendation (Biorad, U.S.A). Focusing was carried out at 20 °C for total 58,900 Volthours in Protean i12 IEF Cell (Bio-Rad, U.S.A). After isoelectric focusing, the IPG strips were equilibrated for 15 min in a buffer consisting of 2% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, 6 M urea and 1.5 M TrisHCl (pH 8.8) containing 2 mM DTT, followed by incubation in the same buffer supplemented with 5 mM iodoacetamide for 15 min with gentle shaking. The strips were then placed on top of a vertical SDS PAGE (5–15% gradient gel). Agarose(0.5%w/v) was overlayed and second dimension gel was electrophoresed at 42 mA per gel for ˜5 h in a SE600 electrophoresis unit (Hoefer, U.S.A). Following 2D separation, the gels were fixed for two hrs on 40% ethanol and 10% acetic acid. The gels were stained with colloidal coomassie solution as described previously (Dyballa and Metzger, 2009). The stained gels were then scanned with GS-800 densitometer (Biorad, U.S.A). Differential expression of particular spots in control and treated groups were analyzed through PD Quest 2D analysis software. Spots showing significant differential expression were excised manually and processed for identification by MALDI-TOF.

2.5. *Protein identification and database search*

The excised protein spots were subjected to in-gel trypsin digestion as described previously (Mishra et al., 2016). Tryptic fragments were subjected to peptide mass fingerprinting (PMF) using 4800 Proteomic analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Warrington, U.K) in positive reflector mode and with 20,000 V acceleration voltage. The resulting mass spectra were analysed by NCBI non-redundant database (www.ncbi.nlm.nih.gov) using Mascot search engine (www.matrixscience.com/cgi/searchform). The search parameters allowed were taxonomy rattusnorvegicus, peptide tolerance 1.2 Da, MS/MS mass tolerance 0.6 Da, one missed trypsin cleavage, fixed modification at carbamidomethyl (C) and variable modification at oxidation at methionine (M). Default settings were used for other parameters.

2.6. *Western blotting*

Hi and SNi isolated from three rats in each group were processed separately to prepared 10% homogenate in cell lysis buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA) as described previously (Mudawal et al., 2015). Cytosolic and mitochondrial proteins fractions were then prepared according to the method described earlier (Timmons et al., 2011) and processed for western blotting as described previously (Towbin et al., 1979). The primary antibodies employed for western blotting experiments were as follows: Cyt c (1:400, milipore), Bcl2 (1:1000, CST), LC3a/b (1:1000, CST), βactin (1:5000, sigma), VDAC (1:1000, sigma). VDAC and β-actin were used for the normalization of expression of target proteins.

2.7. *DNA laddering/fragmentation assay*

To assess apoptosis, DNA laddering assay was performed using agarose gel electrophoresis. The whole genomic DNA from Hi and SNi isolated from three rats was extracted separately using FlexiGene DNA Isolation Kit (Qiagen, Germany). Extracted DNA samples were electrophoresed on 1% agrose gel containing ethidium bromide and visualized under UV using Versa DOC imaging system (Biorad, USA).

2.8. *Ultrastructural studies*

For ultrastructural studies, twelve rats offsprings (n = 3 per group) were used. Rats were anesthetized with ketamine (30 mg/kg) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by paraformaldehyde (4%) and glutaraldehyde (0.1%) in a 0.1 M PBS. The brain regions, Hi and SNi were dissected out from the perfused brain and cut into fine pieces (approx. 2 mm). Primary fixation of sections was carried out in 2.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.2) for 2 h. Tissue pieces were post-fixed in osmium tetraoxide (1%) for 1–2 h followed by dehydration and embedding in araldite and DDSA medium. The blocks containing tissue were baked at 65 °C for 48 h and cut into thin sections (60–90 nm) using ultramicrotome (Leica EM UC 67). The staining of thin sections was carried out with uranyl acetate and lead citrate (2%) on copper mesh grids. The sections were examined in FEI Technai G2 spirit twin Transmission electron microscope equipped with Gatan digital CCD Camera (Netherland) at an accelerating voltage (80 KV).

2.9. *Statistical analysis*

One-way analysis of variance using the GraphPad Prism software was used to analyze the data. Average of six observations for behavioral and neurochemical parameters and three observations for 2D gel electrophoresis and western blotting were taken for analyzing the data statistically. To assess the levels of significance comparing all the pair of

Table 1

Enzymatic and non-enzymatic antioxidants in brain regions of control and lindane treated offspring.

	Hippocampus				Substantia nigra			
	Con	Pre	Post	Rech	Con	Pre	Post	Rech
LPO	74.3 ± 10	108.9 ± 12	190 ± 21*a*b	210 ± 19**ab	77.4 ± 9	98.8 ± 11	176 ± 18**a**b	227 ± 23**a*b*c
Reduced GSH	0.73 ± 0.07	0.62 ± 0.06	0.36 ± 0.04**a*b	0.20 ± 0.02***a**b*c	0.63 ± .03	0.47 ± 0.05*a	0.31 ± 0.01**a*b	0.21 ± 0.03***a**b*c
SOD	0.39 ± 0.02	0.31 ± 0.03	0.20 ± 0.02*ab	0.13 ± 0.01***a**b*c	0.41 ± 0.03	0.33 ± 0.02	0.22 ± 0.01*a*b	0.16 ± 0.01**a*b*c
Catalase	0.47 ± 0.05	0.32 ± 0.04*a	0.20 ± 0.01*ab	0.10 ± 0.01**a*b*c	0.42 ± .03	0.32 ± 0.03	0.26 ± 0.02*a	0.18 ± 0.01***ab*c
Protein carbonyl	5.71 ± 0.5	6.43 ± 0.7	13.6 ± 1.0**a**b	16.4 ± 1.3***ab*c	5.89 ± .033	7.05 ± 0.68	10.4 ± 0.7**a*b	12.3 ± 0.7**ab*c

Con.- Control; Pre-Prenatally exposed offspring; Post- Postnatally exosed offspring; Rech- Rechallenged offspring.

Values are mean ± S.E.M. of six animals from each group.

LPO: nmoles of MDA formed /min/mg protein. GSH: mmole of GSH-CDNB conjugate/mg of tissue; SOD: nmoles of nitrobluetertrazolium reduction/min/mg protein; Catalase: µmolesH_2O_2 /mg protein / min;Protein carbonyl content: nmoles/mg protein.

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. a - compared to control group; b - compared to prenatally exposed offspring group; c - compared to offspring exposed postnatally with lindane; % calculated as compared to control.

columns, Newman–Keuls test was employed and values up to $p < 0.05$ considered significant. Values have been expressed as the mean ± SEM.

3. Results

3.1. Oxidative stress in brain regions of rechallenged offspring

No significant changes were observed in lipid peroxidation, protein carbonyl, GSH content and specific activity of SOD and catalase in Hi and SNi isolated from offspring prenatally exposed to lindane when compared to the controls. A statistically significant increase in the lipid peroxidation and protein carbonyl associated with a decrease in GSH content and activity of SOD and catalase were observed in Hi and SNi isolated from control offspring treated postnatally at adulthood with lindane when compared to the control (Table 1). A much higher magnitude of increase in lipid peroxidation, protein carbonyl associated with a decrease in GSH content and activity of SOD and catalase were observed in rechallenged offspring when compared to the control offspring treated postnatally with lindane (Table 1).

3.2. Proteome profiling and identification of differentially expressed protein spots in rechallenged rats

2D-PAGE analysis revealed differential expression of a total of 36 and 30 protein spots respectively in Hi and SNi isolated from control offspring or offspring prenatally exposed to lindane or offspring treated postnatally with lindane or rechallenged offspring (Tables 2a–2c). Though classes of proteins altered in Hi and SNi were the same in different treatments, differences were observed in the alterations of proteins within the same class. In general, rechallenged offspring showed a much higher magnitude of downregulation in expression of different proteins followed by control offspring treated postnatally with lindane when compared to prenatally exposed offspring or controls (Fig. 1; Tables 2b, 2c).

The proteomic profiling revealed significant alterations in proteins related to mitochondrial and associated energy metabolism in both brain regions isolated from rechallenged offspring (Tables 2b, 2c). Aconitase hydratase mitochondrial precursor (ACO2), triosephosphateisomerase (TPI), isocitrate dehydrogenase [NADP] cytoplasmic (IDH1), V-type proton ATPase subunit B, brain isotype (ATP6V1B2), transaldolase (TALDO1) were significantly downregulated in both Hi and SNi isolated from rechallenged offspring when compared to the controls. Malate dehydrogenase1 (MDH1), creatine kinase B-type (CKB), NADH dehydrogenase were identified to be downregulated only in Hi while ATP synthase α- subunit precursor (ATP5F1A), succinate dehydrogenase mitochondrial precursor (SDHB) and protein kinase M (PKM) were downregulated in SNi in greater magnitude in rechallenged offspring when compared to the controls. Downregulation, though to a lesser extent, was observed in the expression of these proteins in the studied brain regions in control offspring exposed postnatally to lindane when compared to the controls (Tables 2b, 2c). However, least or no significant change in the expression of these proteins was observed in Hi and SNi isolated from prenatally exposed offspring when compared to the controls.

Similarly, greater magnitude of downregulation of proteins, involved in regulating antioxidant response, such as SOD1, preoxiredoxin-VI/I was observed in SNi and Hi isolated from rechallenged offspring (Tables 2b,2c). However, these proteins were also downregulated, though to a lesser extent, in the studied brain regions in control offspring postnatally exposed to lindane when compared to the controls. Likewise, a significant decrease was observed in the expression of proteasomal subunits such as 26S protease regulatory subunit 7 (PSMD13) and proteasome subunit α type 6 (PSMA-6) in both Hi and SNi while proteasome subunit R delta was significantly decreased in SNi. Proteasome subunit α type 1(PSMA-1), proteasome subunit β type-4 (PSMB-4) and ubiquitin conjugating enzyme E2K (UBE2K) were decreased in Hi of rechallenged offspring when compared to the control offspring. However, these proteins showed least no significant changes in brain regions isolated from prenatally exposed offspring, though lesser magnitude of downregulation was observed in control offspring exposed postnatally to lindane when compared to the controls (Tables 2b,2c).

Further, expression of chaperone proteins such as protein disulfide-isomerase A3 (PDIA-3), endoplasmic reticulum protein 29 (ERP-29), protein deglycase (DJ-1) showed significant downregulation in Hi isolated from rechallenged offspring. The expression of proteins such as endoplasmin precursor (HSP90B1) and HSPA8 showed significant downregulation in SNi isolated from rechallenged offspring when compared to the control offspring. However, CCT3 showed significant downregulation in both SNi and Hi isolated from rechallenged offspring when compared to the control offspring (Tables 2b,2c). Significant alteration, though to a lesser extent, in the expression of the above proteins was observed in control offspring treated postnatally with lindane while lesser or no significant changes were observed in studied brain regions of prenatally exposed offspring when compared to control (Tables 2b,2c).

The expression of structural proteins associated with synaptic assembly and cytoskeletal integrity including neurofilament light chain (NEFL), profilin2 and WD repeat protein 1 (WDR1) were significantly downregulated in both Hi and SNi isolated from rechallenged offspring. Glial fibrillary acidic protein (GFAP) in Hi and neurofilament medium chain in SNi was also significantly downregulated in rechallenged offspring when compared to the controls. Likewise, expression of proteins associated with axonal growth such as dihydropyrimidinase-related protein2 in Hi and SNi also showed significantly higher downregulation

Table 2a
Detection of differentially expressed protein spots in Hi and SNi isolated from control and lindane treated groups by MALDI TOF.

Spot no.	Protein name	Protein symbol	Acession number	Score	% sequence coverage	Peptide match	Mr	PI
1	Neurofilament light polypeptide	NFL	gi|13929098	94	5	3(1)	61335	4.0
2	V-type proton atpase subunit B	ATP6V1B2	VATB2_RAT	264	22	8(8)	56857	5.5
3	Dihydropyrimidinase-related protein 2	DPYSL2	NP_034085.2	128	5	2(2)	62638	5.9
4	T-complex protein 1 subunit gamma	CCT3	NP_954522.1	126	5	2(2)	61179	6.2
5	WD repeat-containing protein 1	WDR1	WDR1_RAT	39	5	2(2)	66824	6.1
6	Rab GDP dissociation inhibitor beta	GDI2	NP_058972.2	322	18	7(7)	51018	5.9
7	Elongation factor 1-gamma	EEF1G	NP_001004223.1	173	10	4(4)	50371	6.3
8	Isocitratedehydrogenase [NADP] cytoplasmic	IDH1	NP_113698.1	242	17	5(5)	42970	5.9
9	Transaldolase 1	TALDO1	EDM12033.1	116	7	2(2)	35745	8.2
10	26S proteasome non-atpaseregulatory subunit 13	PSMD13	NP_001102395.1	385	24	7(7)	43075	5.5
11	Guanine nucleotide-binding protein G(o) subunit alpha	GNAO1	GNAO_RAT	508	22	8(8)	40613	5.3
12	Purine nucleoside phosphorylase	PNP	NP_001099501.1	119	9	2(2)	24492	6.6
13	Proteasome subunit alpha type-6	PSMA6	NP_058979.1	216	18	3(3)	27838	6.3
14	Triosephosphate isomerase	TPI1	AAA42278.1	189	18	3(3)	27417	6.4
15	Peroxiredoxin-6	PRDX6	NP_446028.1	116	26	2(2)	14147	6.5
16	Cu-Zn dismutase, partial	SOD1	CAA29121.1	57	8	1(1)	15740	5.89
17	Profilin 2	PFN2	EDM14874.1	216	11	2(2)	15423	5.7
19	Peroxiredoxin-1	PRDX1	NP_476455.1	190	25	4(4)	22323	8.7
20	Proteasome subunit alpha type-1	PSMA1	NP_058974.1	259	29	5(5)	29784	6.15
21	Endoplasmic reticulum protein 29	ERP29	EDM13737.1	66	9	1(1)	17918	7.7
22	Phosphatidylinositol transfer protein	PITPNA	NP_058927.1	133	13	3(3)	32115	5.9
23	Malate dehydrogenase 1, NAD (soluble)	MDH1B	AAH59124.1	192	12	3(3)	36632	5.9
24	Isocitratedehydrogenase 3 (NAD+) alpha	IDH3A	EDL95540.1	294	19	6(6)	41606	6.4
25	Transaldolase 1	TALDO1	EDM12033.1	57	3	1(1)	35745	8.2
26	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial precursor	NDUFS4	NP_001011907.1	41	4	1(1)	52927	6.5
27	Creatinekinase B-type	CKB	NP_036661.3	141	12	3(3)	42983	5.3
28	Pre-mrna-processing factor 19	PRPF19	NP_598890.1	71	5	2(2)	44075	6.4
29	Aconitate hydratase, mitochondrial precursor	ACO2	NP_077374.2	133	3	2(2)	86121	7.8
30	Protein disulfide-isomerase A3	PDIA3	PDIA3_RAT	105	5	2(2)	57044	5.8
31	Glial fibrillary acidic protein delta	GFAP	AAD01874.2	92	2	1(1)	48809	5.7
32	14-3-3 protein zeta/delta	YWHAZ	NP_035870.1	194	25	4(4)	27925	4.7
33	Proteasome subunit beta type-4	PSMB4	NP_113817.2	206	21	4(4)	22507	5.3
34	Protein deglycase DJ-1 isoform 2	PARK7	NP_476484.1	196	15	4(4)	20190	6.3
35	Nucleoside diphosphate kinase A	NME1	NP_612557.1	418	48	7(7)	17296	5.9
36	Ubiquitin-conjugating enzyme E2 K	UBE2K	NP_005330.1	108	21	3(3)	22507	5.6
37	High mobility group protein B1	HMGB1	NP_034569.1	86	11	2(2)	25049	5.6
38	Neurofilament 3, medium	NEFM	EDL85422.1	644	18	11(11)	95765	4.7
39	Endoplasmin precursor	HSP90B1	NP_001012197.2	254	9	5(5)	74390	5
40	Dnak-type molecular chaperone hsp72	OS11G0703900	pir||S31716	627	29	13(13)	71112	5.4
41	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	NP_079555.1	157	11	4(4)	51230	5.3
42	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor	SDHA	NP_569112.1	127	7	3(3)	61998	6.04
43	Aconitate hydratase, mitochondrial precursor	ACO2	NP_077374.2	118	5	3(3)	86121	7.8
44	Pyruvate kinase PKM	PKM	NP_445749.1	248	13	6(6)	58294	6.6
45	ATP synthase alpha subunit precursor	ATP5F1A	AAA40784.1	68	5	1(1)	25639	7.03
46	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GNB1	GBB1_RAT	257	18	5(5)	38151	5.6
47	Proteasome subunit R-DELTA	PSMB6	BAA01586.1	65	5	1(1)	21864	4.9
48	Phosphatidylethanolamine-binding protein 1	PEBP1	NP_058932.1	460	40	5(5)	20902	5.4

Protein spots numbers 1–19 are differentially expressed in both Hi and SNi. Protein spots numbers 20–37 are differentially expressed only in Hi. Protein spots numbers 38–48 are differentially expressed only in SNi.

in rechallenged rats when compared to control offspring (Tables 2b, 2c). The expression of these proteins also showed significant downregulation in Hi and SNi isolated from control offspring postnatally exposed to lindane when compared to the control offspring, though the magnitude of alterations was lesser when compared to the rechallenged offspring. No significant alteration in the expression of structural proteins was observed in prenatally exposed offspring when compared to the control offspring (Table 2b, 2c).

Similarly, significant downregulation in the expression of proteins involved in signal transduction such as Rab GDP dissociation inhibitor beta (GDI2) and guanine nucleotide binding protein G(0) (GNAO1) was observed in both Hi and SNi isolated from rechallenged offspring when compared to the control offspring. Phosphatidylethanolamine binding protein 1 (PEBP1) and GNB1 in SNi were identified to be downregulated significantly in rechallenged offspring. Phosphatidylinositol transfer protein alpha (PITPNA), 14-3-3 protein ζ (YWHAZ), nucleoside diphosphate kinase A (NME1) were also identified to be downregulated significantly in Hi of rechallenged offspring. Further, proteins related to DNA and mRNA mechanism such as elongation factor 1γ and purine nucleoside phophorylase also showed significant downregulation in expression in Hi and SNi isolated from rechallenged offspring when compared to the control offspring. (Tables 2b, 2c). Pre mRNA processing factor 19 (PRPF19) and high mobility group protein B1 (HMGB1) in Hi and heterogeneous nuclear ribonucleoprotein k (HNRNPK) in SNi showed significant downregulation in expression in rechallenged offspring when compared to the control offspring. No significant downregulation in expression of these proteins was observed in prenatally exposed offsprings or postnatally exposed offsprings in SNi. A significant downregulation, though to a lesser extent was observed in Hi isolated from control offsprings treated postnatally with lindane when compared to control offsprings. However less or no significant downregulation in expression of these proteins was observed in prenatally exposed offsprings (Tables 2b,2c).

Table 2b
Downregulation (Percent change) in expression of identified proteins in SNi of lindane treated offspring when compared to control.

Protein	Pre	Post	Rech
Mitochondrial and energy metabolism			
ATP synthase alpha subunit precursor(ATP5F1A)	16.6 ± 4.0	21.7 ± 3.0	53.0 ± 6.6**
Triosephosphate isomerase(TPI1)	12.2 ± 2.1	20.2 ± 2.7*	39.5 ± 5.5**
Isocitrate dehydrogenase [NADP] cytoplasmic(MDH1B)	4.1 ± 1.1	3.9 ± 1.6	41.8 ± 7.3***
V-type proton ATPase subunit B, brain isoform(ATP6V1B2)	9.9 ± 2.7	5.9 ± 1.2	37.6 ± 5.8***
Succinate dehydrogenase [ubiquinone]mitochondrial precursor(SDHA)	46.0 ± 5.6*	55.1 ± 6.0**	60.0 ± 7.6**
Protein kinase M(PKM)	21.1 ± 3.1	20.2 ± 4.9	54..8 ± 6.9**
Aconitate hydratase, mitochondrial precursor(ACO2)	31.0 ± 4.8*	21.2 ± 2.9	55.3 ± 7.4**
Transaldolase 1(TALDO1)	16.1 ± 2.5	4.8 ± 1.0	51.9 ± 6.3**
Ubiquitin proteasome system			
26 s preotease regulatory subunit 7(PSMD13)	11.1 ± 2.2	17.9 ± 2.1*	35.3 ± 4.0**
Proteasome subunit α type-6(PSMA6)	2.4 ± .0.3	24.4 ± 2.6*	72.5 ± 8.1**
Proteasome subunit R delta(PSMB6)	27.5 ± 3.0*	5.5 ± 1.0	47.2 ± 6.9**
Chaperones			
T-complex protein 1 subunit gamma (CCT3)	38.8 ± 4.4*	48.2 ± 5.7*	86.6 ± 9.0***
Endoplsamic precursor(HSP90B1)	9.8 ± 1.8	16.9 ± 2.7*	31.9 ± 3.8**
Dnak-type molecular Chaperone hsp72(Hspa8)	18.11 ± 2*	9.1 ± 1.3	29.3 ± 3.2*
Oxidative stress			
Peroxiredoxin-6(PRDX6)	20.0 ± 4.5	32.7 ± 4.4*	59.4 ± 6.2**
Cu-Zn superoxide dismutase (SOD1)	25.1 ± 4.5	38.7 ± 4.9*	54.8 ± 7.9**
Peroxiredoxin-1(PRDX1)	23.3 ± 4.0	20.3 ± 3.9	52.9 ± 7.7**
Structural proteins			
WD repeat protein 1(WDR1)	0.008 ± 0.2	25.5 ± 3.2*	79.4 ± 8.7**
Profilin2(PFN2)	4.7 ± .0.8	19.4 ± 2.4	73.4 ± 8.6**
NeFL medium(NEFM)	2.2 ± 0.6	7.4 ± 1.7	48.5 ± 6.7**
NeFL light(NFL)	4.3 ± 0.9	10.2 ± 1.8	36.2 ± .4.1**
Axon guidance			
Dihydropyrimidinase-related protein 2 (DPYSL2)	36.4 ± 4.2*	30.4 ± 4.6*	54.5 ± 5.9**
Signal transduction			
Rab GDP dissociation inhibitor beta(GDI2)	13.9 ± 2.1	12.3 ± 1.3	35.9 ± 5.1**
Phosphatidylethanolamine-binding protein 1(PEBP1)	15.41 ± 1.6	22.13 ± 2.8	83.78 ± 9.2**
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1(GNB1)	1.5 ± 0.3	2.2 ± 0.4	30.2 ± 4.2**
Guanine nucleotide-binding protein Go subunit alpha (GNAO1)	13.3 ± 2.3	11.2 ± 1.5	35.4 ± 4.8**
DNA related proteins			
Purine nucleoside phosphorylase(PNP)	4.1 ± 0.6	4.8 ± 0.8	55.5 ± 6.8**
RNA related proteins			
Heterogeneous nuclear ribonucleoprotein K(HNRNPK)	13.9 ± 2.1	6.4 ± 0.9	47.0 ± 5.8**
Translation related proteins			
Elongation factor 1-gamma(EEF1G)	7.5 ± 1.3	12.2 ± 1.6*	25.4 ± 2.8**

Pre-Prenatally exposed offspring; Post-Postnatally exposed offspring; Rech- Rechallenged offspring.
Peptide mass tolerance (1.2 Da); MS/MS mass tolerance (0.6 Da) All the values are calculated in terms of percentage change mean optical density (M.O.D) ± standard error mean (S.E.M) of three samples per group. Densitomertic analysis was performed using PDQuest 2D analysis software (Basic). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control group.

3.3. *Western blotting analysis*

Western blotting revealed relatively high immunoreactivity against cytochrome C (cytC) in mitochondrial fraction (MF) whereas low immunoreactivity was observed in cytoplasmic fraction (CF) isolated from control offspring. No significant change was observed in the expression of cytC in MF and CF in Hi or SNi isolated from prenatally exposed offspring when compared to the controls (Fig. 2). Lower expression of cytC in MF and high expression in CF was observed in the studied brain regions isolated from control offspring treated postnatally with lindane when compared to the controls (Fig. 2). A much greater magnitude of the decrease in the release of cytC from MF and of the increase in CF was observed in Hi and SNi isolated from rechallenged offspring when compared to the control offspring treated postnatally with lindane (Fig. 2).

Similarly, western blot analysis revealed high immunoreactivity against Bcl_2, an anti-apoptotic protein, in protein samples of whole cell lysate isolated from control offspring. No significant change in expression of Bcl2 was observed in prenatally exposed offsprings when compared to control offsprings or offsprings born to control dams treated with lindane at adulthood (Fig. 2). No significant downregulation was observed in control rats treated postnatally when compared to the controls. However, significantly greater magnitude of downregulation was observed in rechallenged offspring when compared to the control rats. (Fig. 2). Western blotting analysis of protein samples of whole cell lysate against autophagic marker LC3a/b showed no significant changes in any treatment group when compared to the controls (Fig. 2).

3.4. *DNA laddering/fragmentation analysis of apoptosis*

Fig. 3 illustrates the effect of lindane on cellular DNA extracted from Hi and SNi isolated from offspring of different groups. Purified DNA isolated from control offspring appeared on the gel as an intact band (lane 2) with no sign of fragmentation in both brain regions. No significant fragmentation was observed in DNA (Lane 3) extracted from Hi or S Ni isolated from prenatally exposed offspring when compared with control offspring. DNA fragmentation was clearly observed in the DNA (Lane 4) extracted from Hi or SNi isolated from offspring born to control dams treated with lindane at adulthood when compared to control offspring. However, smearing was observed in the DNA (Lane 5) extracted from brain regions of prenatally exposed offsprings rechallenged with lindane at adulthood. The presence of less of genomic DNA at the top of the lanes containing DNA of postnatally treated offspring or rechallenged offspring indicates DNA degradation into smaller fragments ie start of apoptosis process whereas smear like appearance of DNA indicated more number of cells undergoing necrosis.

Table 2c
Downregulation (Percent change) in expression of identified proteins in Hi of lindane treated offspring when compared to control.

Protein	Pre	Post	Rech
Mitochondrial and energy metabolism			
Aconitate hydratase, mitochondrial precursor (ACO2)	17.7 ± 2.8	33.5 ± 5.3*	43.1 ± 4.6**
V-type proton ATPase subunit B, brain isoform (ATP6V1B2)	6.8 ± 0.9	24.0 ± 3.3*	58.9 ± 7.3***
Triosephosphate isomerase(TPI1)	19.2 ± 2.5	25.1 ± 4.6*	34.0 ± 5.8**
Isocitratedehydrogense 3(NAD+) alpha, isoform (IDH3A)	13.1 ± 1.9	33.4 ± 4.0*	69.3 ± 8.9**
Malate dehydrogenase 1(MDH1B)	5.5 ± 0.9	26.3 ± 2.9*	33.6 ± 4.1*
Creatine kinase B-type(CKB)	17.4 ± 2.0	40.8 ± 5.2**	70.8 ± 8.6***
Transaldolase 1(TALDO1)	35.4 ± 3.8*	45.3 ± 4.2**	82.3 ± 9.4***
Isocitrate dehydrogenase(IDH1)	25.8 ± 3.1	30.3 ± 4.4*	82.0 ± 9.9***
Transaldolase 1(TALDO1)	23.9 ± 3.3*	19.9 ± 3.1	76.3 ± 8.5***
NADH dehydrogenase(NDUFS4)	51.1 ± 6.2*	55.7 ± 5.9*	87.9 ± 9.7**
Ubiquitin proteasome system			
26 s preotease regulatory subunit 7(PSMD13)	26.9 ± 3.6	44.5 ± 6.2**	74.0 ± 8.8***
Proteasome subunit α type-1(PSMA1)	20.2 ± 2.5	42.4 ± 5.1**	76.8 ± 8.0***
Proteasome subunit β type-4(PSMB4)	25.2 ± 3.1	43.7 ± 5.3**	93.7 ± 9.9***
Ubiquitin conjugating enzymeE2 K isoform 1(UBE2K)	20.0 ± 2.4	50.0 ± 7.4**	77.2 ± 8.6***
Proteasome subunit α type-6(PSMA6)	26.5 ± 3.0*	15.3 ± 1.9	36.4 ± 5.4**
Chaperones			
Protein deglycase(DJ-1)	8.4 ± 1.2	22.5 ± 3.2*	47.0 ± 5.3*
Protein disulfide-isomerase A3(PDIA3)	12.5 ± 2.5	46.5 ± 5.2**	63.9 ± 4.0***
Endoplasmic reticulum protein 29(ERP29)	37.4 ± 5.5*	59.8 ± 8.0*	68.1 ± 7.9**
T-complex protein 1 subunit gamma (CCT3)	38.8 ± 4.5*	37.6 ± 4.8*	55.7 ± 6.5**
Oxidative stress			
Cu-Zn superoxide dismutase (SOD1)	27.2 ± 3.9*	36.2 ± 4.9*	58.2 ± 6.2**
Peroxiredoxin-1 (PRDX1)	34.3 ± 5.6*	44.4 ± 6.7*	47.9 ± 5.0*
Peroxiredoxin-6 (PRDX6)	23.6 ± 4.1*	14.9 ± 1.7	66.3 ± 8.1***
Structural proteins			
WD repeat protein 1 (WDR1)	18.4 ± 2.5	44.8 ± 4.9*	63.9 ± 5.4**
NFL LIGHT (NFL)	8.0 ± 1.1	36.5 ± 4.2*	60.6 ± 6.5**
Profilin2 (PFN2)	9.6 ± 1.5	13.4 ± 1.9	54.3 ± 7.0***
Glial fibrillary acidic protein (GFAP)	27.6 ± 4.4	57.4 ± 6.8**	71.5 ± 8.4***
Axon guidance			
Dihydropyrimidinase-related protein 2 (DPYSL2)	13.2 ± 1.6	24.5 ± 4.4*	52.2 ± 6.5***
Signal transduction			
Phosphatidylinositol transfer protein alpha (PITPNA)	16.6 ± 2.0	39.5 ± 4.4**	73.8 ± 8.4***
Guanine nucleotide-binding protein G(o) subunit alpha (GNAO1)	1.07 ± .3	19.5 ± 2.4*	51.0 ± 7.1***
Rab GDP dissociation inhibitor beta (GDI2)	16.0 ± 2.3*	17.8 ± 1.9 *	38.4 ± 5.6**
14-3-3 protein zeta/delta (YWHAZ)	2.3. ± 0.3	3.9 ± 1.0	30.5 ± 4.4**
Nucleoside diphosphate kinase A (NME1)	34.8 ± 4.2*	43.3 ± 5.7*	78.8 ± 8.7**
RNA related proteins			
Pre-mrna-processing factor 19 isoform 2 (PRPF19)	11.2 ± 1.8	22.9 ± 2.5*	59.7 ± 7.8**
DNA related proteins			
High mobility group protein B1 (HMBG1)	14.8 ± 1.5	49.2 ± 5.3**	60.3 ± 8.8***
Purine nucleoside phosphorylase (PNP)	33.0 ± 5.4*	43.0 ± 6.0*	79.1 ± 9.1**
Translation related proteins			
Elongation factor 1-gamma (EEF1G)	28.9 ± 3.3*	27.4 ± 5.6*	47.3 ± 5.0**

Con- Control; Pre-Prenatally exposed offspring; Post- Postnatally exosed offspring; Rech- Rechallenged offspring.
Peptide mass tolerance (1.2 Da); MS/MS mass tolerance (0.6 Da) All the values are calculated in terms of percentage change mean optical density (M.O.D) ± standard error mean (S.E.M) of three samples per group. Densitomertic analysis was performed using PDQuest 2D analysis software (Basic).$^{*}p < 0.05$, $^{**}p < 0.01$ compared to control group.

3.5. Neurobehavioral activity in rechallenged offspring

Spontaneous locomotor activity (SLA) test revealed no significant changes in the parameters of SLA in prenatally exposed offspring when compared to the control offspring. Significant increase in resting time and stereotypic count associated with decrease in distance traveled, moving time and rearing was observed in control offspring treated postnatally at adulthood when compared to the control offspring (Table 3). Relatively higher magnitude of increase was observed in resting time along with a decrease in distance traveled, moving time, stereotypic count and rearing in rechallenged offspring when compared to the control offspring (Table 3).

Significant reduction in percent alterations were observed in control rats postnatally treated with lindane when compared to the control offspring while no significant changes were observed in percent alterations in prenatally exposed offspring. A much higher and significant decrease in percent alterations was observed in rechallenged offspring when compared to control offspring treated postnatally with lindane (Table 4).

3.6. Ultrastructural alterations induced by lindane in hippocampus and substantia nigra isolated from rechallenged rats

Ultrastructural analysis of Hi and SNi of control offspring exhibited normal morphology at the subcellular level such as clear nucleus with uniform chromatin and nucleoli, mitochondria with clear packed cristae, normal synaptic and myelin structure (Fig. 4a–e). Ultrastructural analysis of Hi and SNi isolated from prenatally exposed rats revealed moderately distorted mitochondria and demyelinated neurons in the studied brain regions. However, no significant changes in the synapse and chromatin in the nucleus were observed in Hi and SNi isolated from prenatally exposed offspring when compared to the controls. Autophagic vesicles with damaged cytoplasmic contents surrounded by normal cellular structure were observed in prenatally exposed offspring (Fig. 4a–e).TEM analysis of Hi and SNi of control offspring exposed postnatally to lindane revealed swollen mitochondria

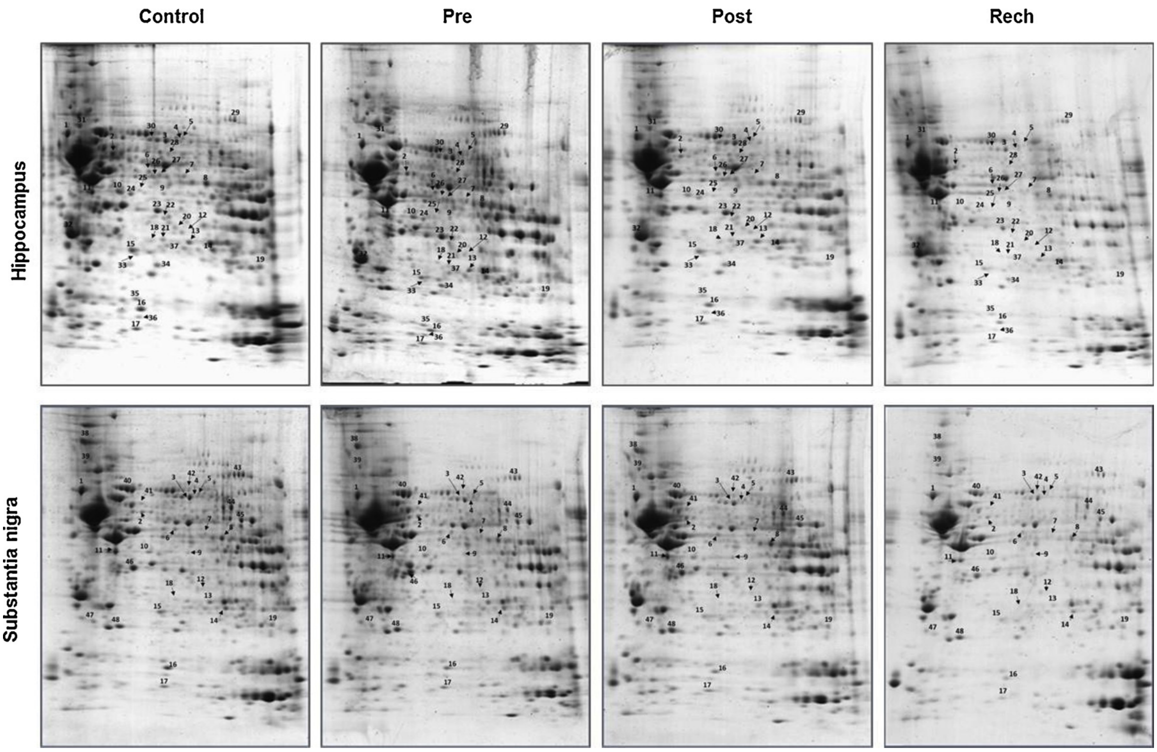

Fig. 1. Representative 2-PAGE profile of hippocampus and substantia-nigra isolated from control, lindane prenatally exposed (Pre), lindane postnatally exposed (Post) and rechallenged (Rech) rat offspring at adulthood (12 weeks). Numbers indicate differentially expressed protein spots.

with disintegrated cristae along with shortened and fuzzy synapse, disintegrated myelin layer and well-developed autophagosome surrounded by shrinked and damaged cytoplasmic material in both the brain regions when compared to the controls (Fig. 4a–e). Rechallenge of lindane caused a much greater extent of loss of mitochondrial integrity with loss of cristae and number in association with severe loss of synaptic structure. Severe demyelination with highly condensed nucleus and cytoplasmic content showing the necrotic effects was observed in both Hi and SNi isolated from rechallenged offspring. Due to massive damage including necrotic changes at the subcellular level, no autophagosome like structure were observed in rechallenged offspring (Fig. 4a–e).

4. Discussion

The present proteomic data has shown that though prenatal exposure of low dose of lindane did not induce any significant effect in the expression of the cerebral proteins related to energy metabolism,

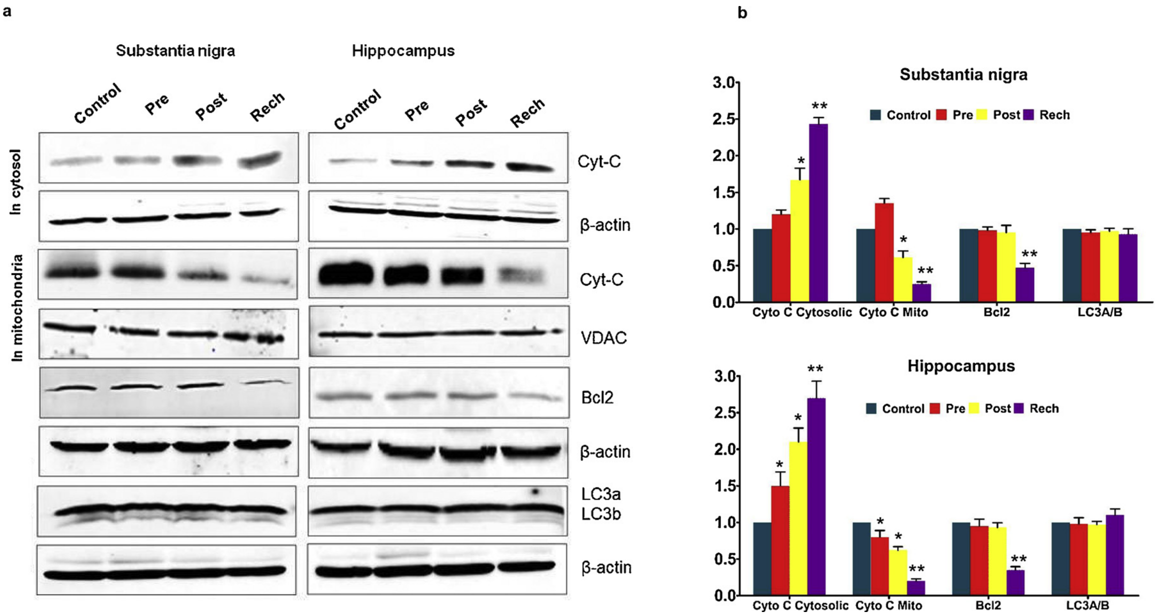

Fig. 2. Representative western blots of protein involved in apoptosis, isolated from substantia nigra and hippocampus isolated from control and lindane prenatally exposed (Pre), lindane postnatally exposed (Post) and rechallenged (Rech) offspring. All the figures represent three experiments (n = 3) in each group; * $P < 0.05$. ** $P < 0.01$.

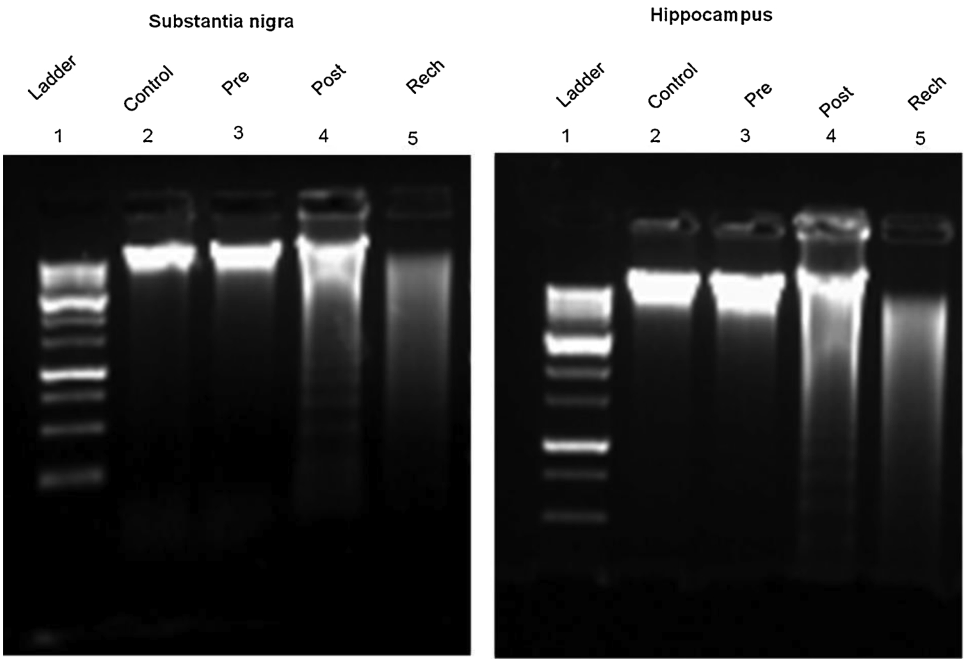

Fig. 3. DNA laddering of hippocampus and substantia nigra of control (Lane2), lindane prenatally exposed (Pre, Lane3), lindane postnatally exposed (Post, Lane4) and rechallenged rats (Rech, Lane5). Lane 1-1 Kb ladder. The experiment was performed with brain region isolated from three rats from each control and treatment group at 12 weeks. All the figures represent three experiments (n = 3) in each group.

mitochondrial functions, chaperons, unfolded protein response (UPR) and apoptosis, enhanced responsiveness of proteins involved in these processes was observed in Hi and SNi isolated from offspring rechallenged at adulthood. The greater magnitude of alterations observed in the mitochondrial proteins such as ATP6V1B2, ACO2, SDHB, TPI1, CKB, TALDO1, IDH1, IDH3A and ATP5F1A, DJ-1, SDHA, PKM, MDH1B in Hi or SNi isolated from rechallenged offspring could be attributed to the persistent biochemical changes in the nervous system in offspring exposed during gestation which are unmasked following subsequent exposure at adulthood. (Barlow et al., 2007; Kraft et al., 2016) Prenatal and early perinatal stress including exposure to pesticides or low doses of gamma radiation is reported to induce mitochondrial dysfunction in the offspring that triggers persistent alterations in the proteome particularly the proteins associated with synaptic impairment.(Bonvallot et al., 2018; Kempf et al., 2014) That these persistent changes lead to increased oxidative stress was demonstrated by significantly higher magnitude of increase in the lipid peroxidation, depletion of reduced glutathione content and increase in the expression of antioxidant enzymes in brain regions of rechallenged offspring when compared to control offspring treated postnatally with lindane. Ultrastructural analysis is in support of the proteomic data indicating severe loss of mitochondrial integrity in the Hi or SNi isolated from rechallenged offspring. Previous reports have also shown that prenatal exposure to pesticides and pesticide mixtures disrupts mitochondrial functions leading to increased oxidative stress in the brain which results in persistent changes in pathways important in neurodegenerative diseases in offspring (Bonvallot et al., 2018; Knopik et al., 2019)

Table 4
Y-maze activity of control and lindane treated offspring.

Group	Percent alterations
Con	55.3 ± 5.8
Pre	42.2 ± 4.9
Post	34.9 ± 3.6^{*a}
Rech	24.8 ± 2.9^{***a*b}

Con.- Control; Pre-Prenatally exposed offspring; Post-Postnatally exposed offspring; Rech-Rechallenged offspring.
The values are mean ± S.E.M of six animals from each group.
Significant difference have been considered up to *p < 0.05 and ***p < 0.001. a - compared to control group; b –compared to prenatally exposed offspring group.

Further evidence that prenatal exposure of lindane induces persistent proteome alterations was provided in the present study demonstrating significantly higher magnitude of downregulation in the expression of chaperon proteins and proteins related to ubiquitin proteosome pathway (UPP) in Hi and SNi isolated from rechallenged rats when compared to compared to control offspring treated postnatally at adulthood. Previous studies from our laboratory have shown that lindane induces oxidative stress leading to UPR in brain regions isolated from rats of different ages by dysregulating UPP (Mudawal et al., 2015, 2018). Romero-Granados et al. (2015) have also reported that early

Table 3
Spontaneous locomotor activity of control and lindane treated offspring.

Group	Distance travelled	Resting time	Moving time	Number of rearing	Stereotypic count
Con	5341.6 ± 820.7	144.2 ± 9.9	131.8 ± 14.4	21.3 ± 3.2	48.6 ± 5.1
Pre	4878.3 ± 692.4	140.7 ± 10.3	135.3 ± 15.1	17.3 ± 2.0	37.8 ± 5.2
Post	2900 ± 643.1^{*ab}	182.8 ± 12.1^{*ab}	93.2 ± 8.0^{*ab}	11.5 ± 1.52^{*a}	80.5 ± 9.4^{*ab}
Rech	1770 ± 394.5^{**ab}	222.9 ± 13.8^{***ab*c}	53.1 ± 6.5^{***ab*c}	8.1 ± 0.92^{*ab}	26 ± 3.2^{*a*c}

Con- control; Pre-Prenatally exposed offspring; Post- Postnatally exosed offspring; Rech- Rechallenged offspring.
The values are mean ± S.E.M of six animals from each group.
Significant difference have been considered up to *p < 0.05, **p < 0.01 and ***p < 0.001. a - compared to control group; b -compared to prenatally exposed offspring group; c - compared to offspring exposed postnatally with lindane.

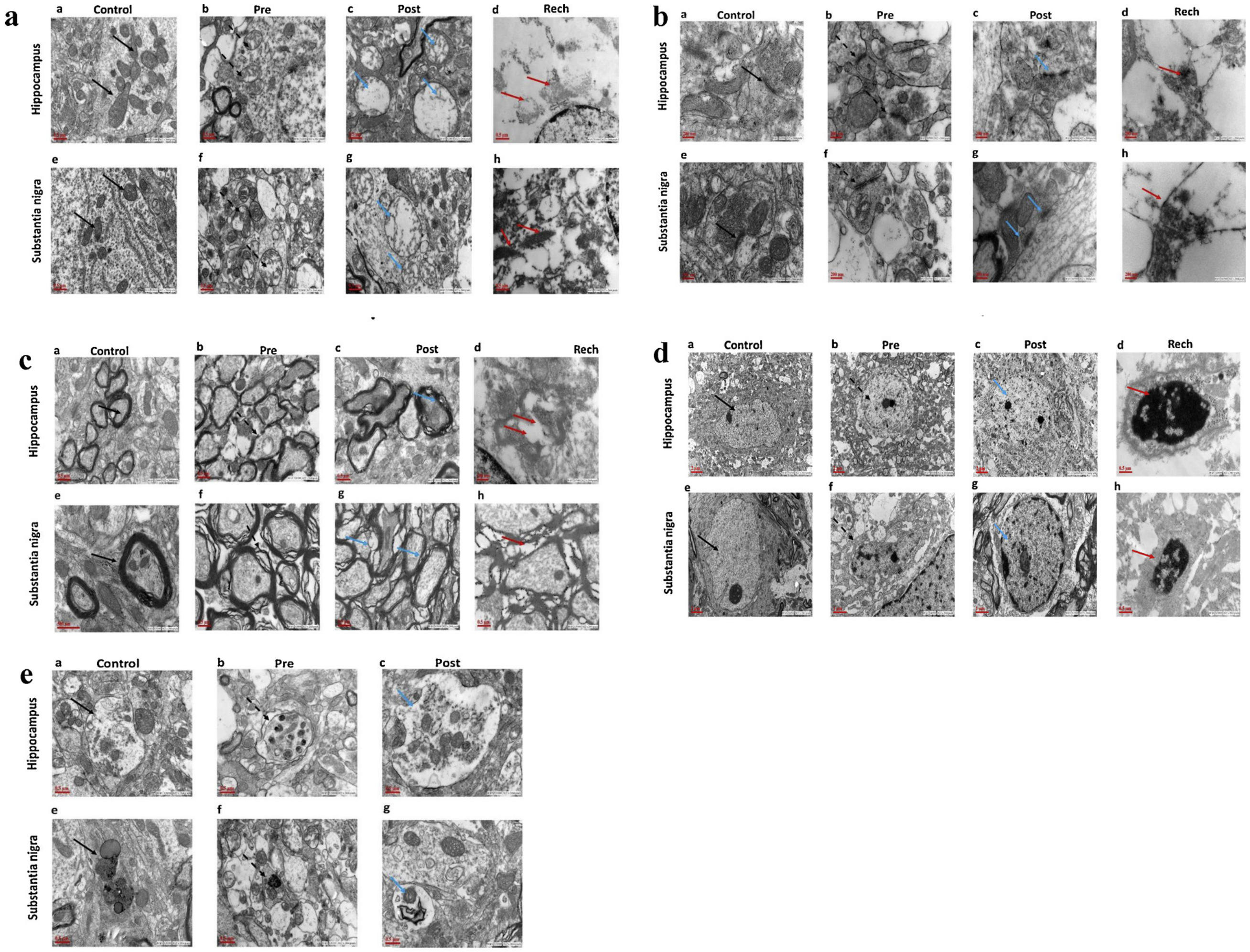

Fig. 4. **a**: Representative transmission electron microscopy (TEM) images revealing mitochondrial morphology in hippocampus (Hi) and substantia nigra (SNi). Hi and SNi of control rats showing intact and healthy mitochondria (a,e; black arrow). Hi and SNi of prenatal group showing disintegrated mitochondria (b,f; broken arrow). Hi and SNi of postnatal group indicate mitochondrial swelling and loss of cristae (c,g; blue arrow). Hi and SNi of rechallanged rats showing severe loss of mitochondrial integrity and number (d,h; red arrow). **b**: Representative transmission electron microscopy (TEM) images of synapse in hippocampus (Hi) and substantia nigra (SNi). Hi and SNi of control rats showing clear pre and postsynaptic membrane, synaptic vesicles (a,e; black arrow). Hi and SNi of prenatal group showing moderately distorted synapse(b,f; broken arrow). Hi and SNi of postnatal group indicate shortened and fuzzy postsynaptic density (c,g; blue arrow). Hi and SNi of rechallanged rats showing severe loss of synapse and synaptic vesicles (d,h, red arrow). **c**: Representative transmission electron microscopy (TEM) images of myelination in hippocampus (Hi) and substantia nigra (SNi). Hi and SNi of control rats showing intact myelin sheath (black arrow, a, e). Hi and SNi of prenatally exposed rats showing slightly disruption in myelin layers (b,f; broken arrow). Disintegrated myelin also appeared on Hi and SNi of postnatally exposed rat (c,g; blue arrow)). Severe degradation with loss of myelin in Hi and SNi of rechallanged rats (d,h; red arrow). **d**: Representative transmission electron microscopy (TEM) images of nuclear morphology in hippocampus (Hi) and substantia nigra (SNi). Hi and SNi of control, prenatal and postnatally exposed rats showing clear continuous nuclear membrane and nucleolus (a,e black arrow; b,f broken arrow; c,g blue arrow). Hi and SNi of rechallanged rats showing severe loss of disrupted nuclear morphology with highly condensed chromatin material (d,h; red arrow). Fig. 4e Representative transmission electron microscopy (TEM) images of autophagy like structures in hippocampus (Hi) and substantia nigra (SNi). Hi of control and prenatal rats showing normal autophagic feature with degraded cellular material in (a, black arrow; b, broken arrow). Postnatally exposed rats showing accumulated autophagic vesicles like structure containing non degraded cellular material (c, blue arrow). SNi of control rats showing secondary lysosome in (e; black arrow). SNi of prenatally exposed rats showing normal autophagic feature (f, broken arrow). SNi of postnatally exposed rats showing accumulation of autophagic vesicles like structures (g, blue arrow). Due to severe distruction at subcellular level no autophagic features were observed in Hi and SNi of rechallanged rats. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

postnatal proteasome inhibition in UPR eventually accounts for neurodegeneration and cognitive deficits in adult mice. Downregulated expression of chaperon proteins and proteins involved in UPR have also been linked with increased protein misfolding and aggregation in aging human brain postmortem samples from AD, PD and DS patients and disease models of neurodegeneration suggesting enhanced proteome alterations in rechallenged offsprings and neurodegenerative diseases. (Andreu et al., 2012; Chang et al., 2013; Halloran et al., 2013; Sato et al., 2005)

Ultrastructural analysis demonstrating significantly marked disruption in synaptic structure, demyelination and axons even showing synapsis in necrotic stage is consistent with the proteomic data revealing significantly greater magnitude of downregulation of proteins related to axonal growth (DPYSL 2) as well as structural proteins (WDR1, PFN2, NeFL, NeFM, GFAP) in Hi and SNi isolated from rechallenged rats. Previous studies have shown that prenatal and postnatal exposure of endocrine disruptors including lindane disrupts myelination potential which is associated with impaired learning and memory abilities in the rats. (Mudawal et al., 2018; Tiwari et al., 2012) Though postnatal treatment of lindane affected myelination in both SNi and Hi in control offspring as reflected by significant impairment in learning and memory, much more marked and necrotic effects observed in myelination in these brain regions of rechallenged offspring may explain the greater magnitude of impairment in learning and memory in rechallenged offspring.

Further, our study demonstrating significantly greater DNA fragmentation and release of CytC associated with the highly condensed nucleus and cytoplasmic contents showing apoptotic nuclei and necrotic effects in both, SNi and Hi of rechallenged offspring, is suggestive of the greater magnitude of apoptosis in these offspring when compared to control offspring treated postnatally with lindane. A recent study from our laboratory indicating alterations in the expression of pro- and anti-apoptotic proteins and ultrastructural changes has also shown induction of apoptosis in Hi and SNi of adult rats treated postnatally with lindane. (Mudawal et al., 2018) Likewise, no marked changes in expression of LC3 and presence of autophagosome with mitochondria and disrupted myelin, surrounded by shrinked matrix and damaged axon in brain regions of postnatally treated offspring is suggestive of disruption in autophagic process and induction of apoptosis as demonstrated earlier in adult rats treated with lindane. (Mudawal et al., 2018) However, no marked change in the expression of LC3b along with the absence of autophagosome like structures suggests the failure of autophagy to rescue the massive cellular stress because of necrosis induced in the brain following rechallenge of lindane at adulthood. It has been shown that endosulfan and zineb, either individually or in combination induce neuronal cell death via apoptotic and necrotic pathways in SH-SY5Y human neuroblastoma cells. (Jia and Misra, 2007)

In conclusion, the present study has shown that rechallenge of lindane to offspring at adulthood results in significantly increased oxidative stress that leads to mitochondrial dysfunction and massive destruction at subcellular level as revealed by alterations in the whole proteome and TEM analysis of the brain regions. The study further assumes significance as exposure to pesticides including lindane is reported in pregnant women in the agriculture fields at comparable levels, that itself may not manifest any significant change but induce persistent cellular and molecular changes in offspring, which may get unmasked resulting in neurodegeneration, following subsequent exposure at adulthood.

Contribution of authors

AS, SY, and DP planned the study and prepared the manuscript. AS performed 2D electrophoresis with MK. AS and AA also performed studies related to oxidative stress and neurobehaviour. PK and MM were involved in Mass spectrometric studies. AKS performed western blotting studies. JS carried out electron microscopy studies.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the Director from CSIR-IITR lucknow for providing support to the study. The authors also gratefully acknowledge the financial support provided by CSIR-Network Project INDEPTH (BSC0111) and institutional funds. AS is thankful to ICMR for providing Junior and Senior Research Fellowship. IITR Communication Number: 3566.

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