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Silymarin- and melatonin-mediated changes in the expression of selected genes in pesticides-induced Parkinsonism

Naveen Kumar Singhal · Amit Kumar Chauhan · Swatantra Kumar Jain · Rishi Shanker · Chetna Singh · Mahendra Pratap Singh

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Abstract Parkinson's disease (PD) is the second most unconcealed neurodegenerative disorder labelled with motor impairments. Two pesticides, manganese ethylene-1,2-bisdithiocarbamate (maneb) and 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat), together, are reported to increase the incidence of PD in humans and Parkinsonism in mice. Conversely, silymarin and melatonin, two naturally occurring antioxidants, rescue from maneb- and paraquat-induced Parkinsonism. The study examined silymarin- and melatonin-mediated changes in the expression of selected genes in maneb- and paraquat-induced Parkinsonism employing mouse discover chips microarrays. The mice were treated intraperitoneally (i.p.), daily, with silymarin (40 mg/kg) or melatonin (30 mg/kg) for 9 weeks along with vehicles. Subsets of animals were also treated with maneb (30 mg/kg; i.p.) and paraquat (10 mg/kg; i.p.), twice a week, for 9 weeks. Whilst the expression of genes in the striatum was determined by microarray, the expression of randomly selected transcripts was validated by quantitative real-time polymerase chain reaction (qRT-PCR). Combined maneb- and paraquat-treatment altered the expression of several genes associated with apoptosis, inflammation, cell cycle, cell-signalling, etc. pathways. Silymarin and melatonin significantly resisted the changes in the expression of a few genes related to apoptosis, inflammation, cell cycle, cell-signalling, etc. The expression patterns of seven randomly selected genes were analyzed by qRT-PCR, which were found to follow the similar trends, as observed with microarray. The results obtained from the study thus demonstrate that despite resemblances, silymarin and melatonin differentially offset maneb- and paraquat-induced changes in transcriptome.

Keywords Parkinson's disease · Maneb · Paraquat · Silymarin · Melatonin · Microarray

Introduction

Parkinson's disease (PD) is a complex neurological disorder, characterized by the striatal dopamine deficiency, nigrostriatal dopaminergic neurodegeneration and motor impairments [1, 2]. PD is mainly linked with the advanced age but the roles of environmental exposure to pesticides and genetic makeup of an individual have been lately appreciated [1–3]. Maneb, a fungicide, and paraquat, an herbicide, have been implicated in PD pathogenesis through epidemiological and animal studies [1–7]. Maneb crosses the blood–brain barrier owing to its lipophilic nature while paraquat crosses it through the neutral amino acid transporter [2, 3, 7]. Maneb is reported to inhibit the mitochondrial complex III in a few reports but most of the studies did not observe any change in complex III activity. On the other hand, paraquat is consistently reported to inhibit the mitochondrial complex I [2, 3, 5, 7]. These two pesticides together induce more pronounced oxidative

Naveen Kumar Singhal and Amit Kumar Chauhan contributed equally to this work.

N. K. Singhal · A. K. Chauhan · R. Shanker · C. Singh · M. P. Singh (✉)
CSIR-Indian Institute of Toxicology Research, Mahatma Gandhi Marg, Post Box 80, Lucknow 226 001, Uttar Pradesh, India
e-mail: singhmahendrapratap@rediffmail.com

A. K. Chauhan · C. Singh
Academy of Scientific and Innovative Research, New Delhi, India

S. K. Jain
Jamia Hamdard, New Delhi 110 062, India

stress and neurodegeneration than either alone [3, 7]. Combined maneb and paraquat model of PD is widely accepted since it resembles sporadic PD owing to slow and progressive degeneration of dopaminergic neurons and is also environmentally relevant [3, 5]. Due to noteworthy contribution of oxidative stress, neuroprotective potentials of a few antioxidants are assessed in rodent models of PD [4, 8, 9]. Antioxidants, which are derived from natural sources and possessing negligible toxicities and potent anti-inflammatory, free-radical scavenging and anti-apoptotic properties, have been the foremost choices for such investigations [4, 7–9]. Silymarin (3,5,7-trihydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-7-yl]-2,3-dihydrochromen-4-one) and melatonin (*N*-acetyl-5-methoxytryptamine), two naturally occurring agents, are found to rescue from maneb- and paraquat-induced Parkinsonism in mice [4]. While melatonin mainly regulates circadian rhythm and silymarin is a well-known hepatoprotective agent, they can also cross the blood–brain barrier, enter the brain and exert neuroprotective effects [7, 8, 10, 11].

Parkinson's disease pathogenesis is a complex phenomenon, which involves multiple molecular events that include apoptosis, inflammation, oxidative stress, etc. [12–14]. A complex interaction of the cellular and molecular events could lead to dopamine depletion and nigrostriatal dopaminergic neurodegeneration, which eventually lead to motor impairments and thereby PD [15]. DNA microarray is used to identify the differentially expressed transcripts in pesticides-induced PD phenotype [16–18]. The phenotypic features of PD are often taken into account not only just to correlate the expression patterns of the selected genes (transcripts) but also to infer their possible roles in disease pathogenesis/protection [16–18]. Furthermore, the microglial activation is reported to be critical along with inflammation in maneb- and paraquat-induced PD phenotype [5]. The microglial activation induces production of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-1β (IL-1β) [19]. Cytokines induce apoptosis in neurons by regulating the levels of proteases and other secondary mediators, such as caspase 8 (casp8), caspase 9 (casp9), B cell lymphoma 2 (Bcl2), Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) [20–23]. Whilst silymarin and melatonin rejuvenate antioxidant defence system, reduce inflammation, regulate apoptosis and offer neuroprotection against maneb- and paraquat-induced Parkinsonism [4], their effects at the level of multiple genes, which could help in deciphering the unambiguous underlying molecular mechanisms, have not yet been deciphered. The present study was undertaken to assess the multiple gene expression in silymarin- and melatonin-mediated neuroprotection in order to predict the molecular mechanisms involved therein.

Materials and methods

Chemicals

Maneb, paraquat, agarose, diethyl pyrocarbonate (DEPC), bromophenol blue, chloroform, ethidium bromide, ethylenediaminetetraacetic acid, formaldehyde, isopropanol, melatonin, 3-(*N*-morpholino) propanesulphonic acid, sodium citrate, sodium acetate, sodium chloride, sodium dodecyl sulphate (SDS), tri reagent, SYBR green master mix and forward and reverse primers for quantitative real-time polymerase chain reaction (qRT-PCR) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Silymarin was purchased from ICN Biomedical, Irvine, CA, USA. Reverse transcriptase-polymerase chain reaction (RT-PCR) kit and dNTPs were purchased from Fermentas, Vilnius, Lithuania. The cDNA direct labelling kit, oligo-dT, DEPC-treated water, dithiothreitol, reverse transcriptase buffer and enzyme, alexa fluor-555 dye and RNAse-OUT were purchased from Invitrogen, Carlsbad, CA, USA. The discover chips (mouse) microarrays and blocking solution, hybridization buffer and wash buffers A, B and C were purchased from ArrayIt, Sunnyvale, CA, USA. All other chemicals were purchased locally from Sisco Research Laboratories, Mumbai, India or Bangalore Genei, Bangaluru, India.

Animal treatment

Male Swiss albino mice (20–25 g) were kept under the standard conditions of temperature (22 ± 2 °C), humidity (45–55 %), light:dark cycle (12:12 h) and light intensity (300–400 lx). The experiments were carried out in animal house of the institute. The mice were fed pellet diet and water ad libitum. The study was approved by the institutional ethics committee. The mice were treated intraperitoneally, daily, with silymarin (40 mg/kg) or melatonin (30 mg/kg) for 9 weeks along with controls (vehicles) [4]. Subsets of animals were also treated with maneb (30 mg/kg) and paraquat (10 mg/kg) through the same route, twice a week, for 9 weeks along with respective vehicles (controls) [4]. Maneb and paraquat were administered 2 h after the antioxidant treatment, if scheduled for the same day [4]. The mice were sacrificed via the cervical dislocation, the brain was taken out and the striatum was isolated.

RNA isolation, cDNA preparation and labelling

Total RNA isolation from the striatum and cDNA synthesis and integrity assessment were performed as described elsewhere [12]. The cDNA was labelled according to the protocol provided by the manufacturer of labelling kit. The quality of labelled cDNA in agarose gel electrophoresis was checked under laser scanner while quantification at every step was performed as reported previously [12]. In brief, total RNA was isolated using tri reagent. Polyadenylated RNA, present in total RNA, was directly reverse transcribed into alexa fluor-555-labelled cDNA. The amount of RNA or cDNA was estimated by taking the absorbance at 260 nm. The integrity of RNA was determined by measuring the ratio of absorbance at 260 and 280 nm, which was never less than 1.8. Furthermore, the integrity of RNA was confirmed by calculating the band density ratio of 28S and 18S RNA, which was never less than 2.0.

Hybridization and scanning

Mouse discover chips arrays were activated by washing with 2× saline sodium citrate (SSC; pH-7.0) buffer containing 0.1 % SDS, 2× SSC buffer and 0.1× SSC buffer for 5, 5 and 1 min, respectively. The microarray slide was placed in rack and incubated in boiling water bath for 1 min followed by 10 s in absolute ethanol. The slides were incubated in blocking solution for 1 h, washed with deionised water for 1 min and dried under the slide drier. An equal amount (~2 μg) of labelled cDNA was taken from control or treated group and mixed separately with hybridization buffer in the ratio of 1:4. The control and treated cDNAs were hybridized with discover chips and incubated at 42 °C for 18 h. The hybridized discover chips were washed with 3 types of washing buffers each for 5 min. The slides were scanned under laser scanner (Microarray scanner: GenTAC LS IV, Genomic Solutions, USA).

Data analysis

Analysis was done employing Array Vision 8.0 (GE Healthcare, Europe), hierarchical clustering was performed using Cluster_vers_2.11 and tree view was generated employing TreeView_vers_1.60 software (trial version available free of cost). The spots were individually quantified; local backgrounds were calculated from the corners between the spots and signal intensity for each spot was determined by subtracting the background from intensity. The fold changes for the differentially expressed transcripts were calculated from the ratio of intensities of the two test groups. The involvement of molecular pathways is proposed with the help of freely online available software namely "gene map annotator and pathway profiler (GenMAPP) version 2.1" [24]. This software is commonly used to analyze microarray data and to predict the roles of various signalling event. During analysis, the decisive factors were employed, as reported elsewhere [11–13, 24]. Inferences for the functional annotation of genes were derived from http://ncbi.nlm.nih.gov and http://smd.stanford.edu/cgibin/source/sourceBatchSearch websites.

qRT-PCR

The primers were synthesized based on the sequences extracted from the online primer bank database (http://pga.mgh.harvard.edu/primerbank/index.html) (Table 1). The expression patterns of Bcl2-antagonist/killer 1 (Bak1), v-akt murine thymoma viral oncogene homologue 1 (Akt1), IL-1β, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFκB1), heat shock transcription factor 1 (Hsf1), synaptotagmin 5 (Syt5) and casp9 were checked through qRT-PCR (iCycler, Bio-Rad detection system; Bio-Rad, Hercules, CA), as described previously [11]. The values were calculated in terms of cycle threshold (Ct) using ΔΔCt method and are represented in fold changes as compared with controls.

Statistical analysis

The data were analyzed by one-way analysis of variance followed by Newman–Keuls test. The values are expressed in means ± standard error of means (SEM). The difference was considered statistically significant, if '*P*' value was < 0.05.

Results

Differential expression of genes/transcripts

A total of 67 transcripts were found to be altered in the striatum of maneb- and paraquat-treated mice. Out of 67, the 27 transcripts were found to be upregulated while 40 were downregulated. The differentially expressed transcripts were found to be associated with apoptosis, inflammation, mitochondrial dysfunction, cell signalling, synaptic function, cell cycle, cytoskeleton, cellular metabolism, growth factor receptors, ion channels, etc. Silymarin modulated the expression of 49 while melatonin modulated the expression of 54 transcripts, which were altered significantly after combined maneb- and paraquat-treatment. A total of 36 differentially expressed transcripts

Table 1 Name of the transcripts, primer sequences and expected size of the amplicons in qRT-PCR

S. No.	Gene	Real-time primer sequence	Product size (bp)
1.	Bak1	Forward 5′-TGCCTACGAACTCTTCACCAA-3′	114
		Reverse 5′-TGGTAGACGTACAGGGCCAG-3′	
2.	Akt1	Forward 5′-CCTTTATTGGCTACAAGGAACGG-3′	164
		Reverse 5′-GAAGGTGCGCTCAATGACTG-3′	
3.	IL-1β	Forward 5′-GAAATGCCACCTTTTGACAGTG-3′	116
		Reverse 5′-TGGATGCTCTCATCAGGACAG-3′	
4.	NFκB1	Forward 5′-GGAGGCATGTTCGGTAGTGG-3′	135
		Reverse 5′-CCCTGCGTTGGATTTCGTG-3′	
5.	Hsf1	Forward 5′-CCTGGCCCATACTCAGCTC-3′	192
		Reverse 5′-CTCTTGCTTGACACGGACC-3′	
6.	Syt5	Forward 5′-AACACCAGCTAGGCCGACT -3′	176
		Reverse 5′-CACCTTGGTCTCATGTCGCC -3′	
7.	Casp9	Forward 5′-TCCTGGTACATCGAGACCTTG-3′	109
		Reverse P 5′-AAGTCCCTTTCGCAGAAACAG-3′	
8.	GAPDH	Forward 5′-AGGTCGGTGTGAACGGATTTG-3′	123
		Reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′	

were found to be common between melatonin- and silymarin-treated groups. Silymarin and melatonin per se did not alter the expression of any gene, which was altered in maneb- and paraquat-treated animals.

Transcripts associated with apoptosis

The expression patterns of Bax, death-associated protein 3 (Dap3), Bak1, TNFRSF1A-associated via death domain (Ripk1), tumour necrosis factor receptor super-family, member 6 (Fas), caspase 1 (Casp1), Casp9, tumour protein p53 (Trp53) and NFκB1 were found to be upregulated while the expression of Bcl2 was found to be downregulated in combined maneb- and paraquat-exposed animals as compared with controls. Silymarin or melatonin treatment in maneb- and paraquat-exposed mice restored the expression patterns of Bax, Bcl2, Trp53, Bak1, Fas, Casp1, Casp9 and NFκB1 towards normalcy. The expression of Dap3 was restored only by melatonin while Ripk1 was restored only by silymarin (Table 2).

Transcripts associated with inflammation

The expression patterns of IL-1β, IFN-γ, TNF-α, transforming growth factor beta 1 (TGF-β1), tumour necrosis factor, alpha-induced protein 3 (Tnfaip3) and TNF receptor-associated factor 6 (Traf6) were found to be upregulated in the striatum of combined maneb- and paraquat-induced PD phenotype in mice. Silymarin or melatonin co-exposure in the above treatment group significantly rescued from the altered expression patterns of IL-1β, IFN-γ, TNF-α and TGF-β1. Silymarin co-treatment restored the expression of Traf6 while melatonin co-treatment restored the expression of Tnfaip3 towards basal values (Table 2).

Transcripts associated with the mitochondrial function

Whilst the expression patterns of aldehyde dehydrogenase 5 family member A1 (Aldh5a1), adenosine triphosphatase (ATPase), H+ transporting, lysosomal (vacuolar proton pump) 42kD (Atp6v1c1), acyl-coenzyme A dehydrogenase family member 8 (Acads), cytochrome *c* oxidase subunit VIb (Cox6b1) and ribosomal protein L19 (Rpl19) were found to be downregulated, the expression of ornithine decarboxylase antizyme 2 (Oaz2) was increased in the striatum of maneb- and paraquat-treated mice. Silymarin- or melatonin-treated animals exhibited significant modulations towards normalcy with varying levels of significance except Acads, which was restored only by silymarin and Rpl19, which was restored only by melatonin in the striatum of combined maneb- and paraquat-treated animals (Table 2).

Transcripts associated with synaptic function

The expression patterns of secretory carrier membrane protein 2 (Scamp2), Syt5, vesicle-associated membrane protein 5 (Vamp5), vacuolar protein sorting 45B (yeast homolog) (Vps45) and dynamin 2 (Dnm2) were found to be reduced in the striatum of maneb- and paraquat-treated animals as compared with controls. Silymarin or melatonin treatment restored the expression of Syt5 and Vamp5 genes towards normal levels. Moreover, the expression patterns of Vps45 and Dnm2 were restored only by melatonin and

Table 2 List of the differentially expressed transcripts (1.5 fold or more at least in an experimental condition)

Gene symbol	Unigene ID	Fold change		
		Maneb + paraquat	Silymarin + maneb + paraquat	Melatonin + maneb + paraquat
Apoptosis				
Bax	Mm.19904	2.65 ± 0.13***	1.66 ± 0.03##	1.74 ± 0.04#
Bcl2	Mm.257460	−2.34 ± 0.08***	−1.57 ± 0.02#	−1.66 ± 0.04#
Dap3	Mm.29028	3.24 ± 0.08***	3.38 ± 0.07	1.69 ± 0.05##
Bak1	Mm.2443	3.58 ± 0.11***	2.22 ± 0.08##	2.04 ± 0.06##
Ripk1	Mm.374799	3.80 ± 0.03***	1.59 ± 0.02###	3.47 ± 0.03
Casp1	Mm.1051	4.77 ± 0.13***	2.71 ± 0.05##	1.69 ± 0.02###
Fas	Mm.1626	2.61 ± 0.11***	1.71 ± 0.02#	1.81 ± 0.02#
Casp9	Mm.88829	3.23 ± 0.17***	1.69 ± 0.23##	1.79 ± 0.34##
NFκB1	Mm.256765	2.48 ± 0.06***	1.58 ± 0.12#	1.71 ± 0.09#
Trp53	Mm.222	5.28 ± 0.11***	1.95 ± 0.36###	3.20 ± 0.08##
Inflammation				
IL-1β	Mm.222830	5.68 ± 0.20***	2.04 ± 0.09###	1.77 ± 0.08###
Traf6	Mm.292729	3.46 ± 0.14***	1.62 ± 0.03##	3.63 ± 0.14
IFN-γ	Mm.240327	2.20 ± 0.09***	1.56 ± 0.02#	1.64 ± 0.11#
TNF-α	Mm.1293	2.84 ± 0.13***	1.57 ± 0.04##	1.62 ± 0.06##
TGF-β1	Mm.248380	3.37 ± 0.16***	1.93 ± 0.06##	1.67 ± 0.03###
Tnfaip3	Mm.116683	2.98 ± 0.20***	2.48 ± 0.13	1.86 ± 0.22##
Mitochondrial function				
Aldh5a1	Mm.393311	−6.02 ± 0.33***	−1.74 ± 0.22###	1.67 ± 0.35###
Atp6v1c1	Mm.276618	−3.98 ± 0.11***	−1.78 ± 0.23###	−2.25 ± 0.26##
Acads	Mm.18759	−2.91 ± 0.32***	1.62 ± 0.23###	−2.45 ± 0.10
Cox6b1	Mm.400	−3.45 ± 0.38***	−1.97 ± 0.18##	−1.76 ± 0.04###
Oaz2	Mm.116749	2.44 ± 0.05***	1.74 ± 0.03##	2.60 ± 0.13
Rpl19	Mm.10247	−3.06 ± 0.18***	−3.27 ± 0.28	−1.67 ± 0.22##
Synaptic function				
Scamp2	Mm.286069	−4.56 ± 0.21***	−2.13 ± 0.24###	−4.46 ± 0.13
Syt5	Mm.358663	−4.99 ± 0.63***	−2.78 ± 0.55##	−2.00 ± 0.32###
Vamp5	Mm.440321	−2.51 ± 0.14***	−1.70 ± 0.13##	−1.61 ± 0.15##
Vps45	Mm.263185	−2.48 ± 0.14***	−2.14 ± 0.06	−1.58 ± 0.18##
Dnm2	Mm.433257	−3.29 ± 0.35***	−3.57 ± 0.08	−1.83 ± 0.04###
Protein degradation				
Psen1	Mm.998	3.41 ± 0.20***	3.23 ± 0.16	1.84 ± 0.43##
Ctsl	Mm.930	3.07 ± 0.15***	1.87 ± 0.26##	2.66 ± 0.32
Ubb	Mm.371592	−4.38 ± 0.55***	−1.94 ± 0.23###	−2.52 ± 0.15##
Psmc4	Mm.29582	−3.52 ± 0.12***	−3.75 ± 0.12	−1.79 ± 0.08##
Hsf1	Mm.347444	−3.21 ± 0.17***	−1.73 ± 0.12##	−1.54 ± 0.16##
Hspa1b	Mm.372314	−2.79 ± 0.39***	−1.71 ± 0.11##	−2.51 ± 0.17
Hsp90aa1	Mm.341186	−2.42 ± 0.19***	−2.78 ± 0.09	−1.56 ± 0.11##
Cell signalling				
Raf1	Mm.184163	−6.11 ± 0.10***	−2.30 ± 0.28###	−1.92 ± 0.06###
Stat3	Mm.249934	−5.57 ± 0.29***	−1.87 ± 0.13###	−2.03 ± 0.09###
Stat4	Mm.1550	−5.20 ± 0.25***	−1.76 ± 0.18###	−1.87 ± 0.34###
Creb1	Mm.453295	−3.95 ± 0.12***	−1.55 ± 0.29###	1.86 ± 0.17###
Map2k1	Mm.248907	−3.86 ± 0.15***	−1.95 ± 0.24##	−1.97 ± 0.19##
Map2k6	Mm.14487	3.11 ± 0.22***	1.56 ± 0.08###	1.84 ± 0.23##
Elk1	Mm.405823	−1.86 ± 0.13**	−1.83 ± 0.12	1.81 ± 0.21###

Table 2 continued

Gene symbol	Unigene ID	Fold change		
		Maneb + paraquat	Silymarin + maneb + paraquat	Melatonin + maneb + paraquat
Fos	Mm.246513	3.49 ± 0.17***	1.83 ± 0.12$^{\#\#}$	3.34 ± 0.09
Irs2	Mm.407207	−3.37 ± 0.09***	−3.56 ± 0.14	−1.63 ± 0.21$^{\#\#}$
Akt1	Mm.6645	−4.44 ± 0.12***	−1.66 ± 0.12$^{\#\#\#}$	−1.89 ± 0.19$^{\#\#\#}$
Cell cycle, cytoskeletal network and cellular metabolism				
Gstp1	Mm.299292	−3.36 ± 0.18***	−1.59 ± 0.09$^{\#\#}$	−1.78 ± 0.12$^{\#\#}$
Nos2	Mm.2893	3.07 ± 0.42***	2.34 ± 0.08$^{\#}$	1.89 ± 0.03$^{\#\#}$
Pde1b	Mm.390792	−2.68 ± 0.31***	−2.31 ± 0.06	−1.55 ± 0.15$^{\#\#}$
Pdha1	Mm.34775	−4.17 ± 0.51***	−1.92 ± 0.11$^{\#\#\#}$	−3.87 ± 0.09
Eef1g	Mm.379129	−3.98 ± 0.13***	−1.63 ± 0.08$^{\#\#\#}$	1.62 ± 0.12$^{\#\#\#}$
Cdk2	Mm.111326	−4.19 ± 0.12***	−2.05 ± 0.06$^{\#\#\#}$	−1.72 ± 0.14$^{\#\#\#}$
Calm1	Mm.285993	3.05 ± 0.10***	1.54 ± 0.20$^{\#\#\#}$	2.03 ± 0.15$^{\#\#}$
E2f1	Mm.18036	2.73 ± 0.11***	2.29 ± 0.16	1.60 ± 0.21$^{\#\#}$
Cdkn1a	Mm.195663	2.93 ± 0.32***	1.59 ± 0.12$^{\#\#}$	2.51 ± 0.09
Eif4g2	Mm.185453	−5.33 ± 0.12***	−5.24 ± 0.11	−2.06 ± 0.05$^{\#\#\#}$
Mtap2	Mm.256966	−2.41 ± 0.33***	1.69 ± 0.12$^{\#}$	−1.53 ± 0.18$^{\#\#}$
Mapt	Mm.1287	−2.44 ± 0.31***	1.70 ± 0.22$^{\#}$	−1.63 ± 0.11$^{\#}$
Tubb5	Mm.489593	−2.55 ± 0.12***	−2.14 ± 0.17	−1.55 ± 0.16$^{\#\#}$
Pcdh7	Mm.332387	3.20 ± 0.45***	1.71 ± 0.21$^{\#\#}$	3.39 ± 0.12
Growth factor receptors and ion channels				
Ntrk2	Mm.130054	−2.89 ± 0.13***	−1.57 ± 0.09$^{\#\#}$	1.66 ± 0.16$^{\#\#}$
Igf1	Mm.268521	−7.24 ± 0.93***	−1.83 ± 0.22$^{\#\#\#}$	−1.96 ± 0.23$^{\#\#\#}$
Fgfr3	Mm.6904	2.91 ± 0.13***	1.70 ± 0.21$^{\#\#}$	2.46 ± 0.15
Pdgfra	Mm.221403	3.17 ± 0.24***	2.72 ± 0.18	1.68 ± 0.12$^{\#\#}$
P2ry4	Mm.117118	−5.19 ± 0.18***	−5.36 ± 0.18	−1.95 ± 0.21$^{\#\#\#}$
Grb2	Mm.439649	−2.74 ± 0.11***	−1.71 ± 0.06$^{\#}$	−1.60 ± 0.09$^{\#\#}$
Slc22a2	Mm.17322	−2.35 ± 0.18***	−2.12 ± 0.24	−1.60 ± 0.12$^{\#}$
Clca1	Mm.454553	−4.78 ± 0.44***	−1.87 ± 0.12$^{\#\#\#}$	−4.37 ± 0.31
Itpr1	Mm.227912	−5.74 ± 0.25***	−5.37 ± 0.26	−3.88 ± 0.11$^{\#\#}$

The values are expressed in means ± SEM ($n = 3$). Positive (+) value indicates upregulation while the negative (−) value indicates down-regulation of transcripts. Significant changes are expressed as ** $P < 0.01$ and *** $P < 0.001$ in comparison with controls and $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ and $^{\#\#\#}$ $P < 0.001$ in comparison with maneb- and paraquat-treated groups

Scamp2 was restored only by silymarin in the striatum of maneb- and paraquat-induced Parkinsonism in mice (Table 2).

Transcripts associated with protein degradation

The expression patterns of ubiquitin B (Ubb), proteasome (prosome, macropain) 26S subunit ATPase 4 (Psmc4), Hsf1, heat shock 70 kD protein 6 (Hspa1b) and heat shock 90 kD protein 1 alpha (Hsp90aa1) were decreased but the expression patterns of presenilin 1 (Alzheimer disease 3) (Psen1) and cathepsin L2 (Ctsl) were found to be increased after combined maneb- and paraquat-exposure. The expression patterns of Psen1, Psmc4 and Hsp90aa1 were restored only by melatonin while Ctsl1 and Hspa1b were restored only by silymarin (Table 2).

Transcripts associated with cell signalling

Similarly, expression patterns of v-raf-1 murine leukaemia viral oncogene homologue 1 (Raf1), signal transducer and activator of transcription 3 (Stat3), signal transducer and activator of transcription 4 (Stat4), cAMP-responsive element-binding protein 1 (Creb1), mitogen-activated protein kinase kinase 1 (Map2k1), ELK1, member of ETS oncogene family (Elk1), insulin receptor substrate 2 (Irs2) and Akt1 were reduced while the expression patterns of mitogen-activated protein kinase kinase 6 (Map2k6) and v-fos

FBJ murine osteosarcoma viral oncogene homologue (Fos) were found to be increased in maneb- and paraquat-treated mice striatum. The expression patterns of Elk1 and Irs2 were restored only by melatonin; Fos was restored only by silymarin and remaining transcripts by both agents in the striatum of maneb- and paraquat-treated animals (Table 2).

Transcripts associated with the cell cycle, cytoskeletal network and cellular metabolism

The expression patterns of glutathione S-transferase pi (Gstp1), phosphodiesterase IB calmodulin-dependent (Pde1b), pyruvate dehydrogenase (lipoamide) alpha 1(Pdha1), eukaryotic translation elongation factor 1 gamma (Eef1g), cyclin-dependent kinase 2 (Cdk2), eukaryotic translation initiation factor 4 gamma, 1(Eif4g2), microtubule-associated protein 2 (Mtap2), microtubule-associated protein tau (Mapt) and tubulin beta (Tubb5) were found to be downregulated while the expression patterns of nitric oxide synthase 2A (Nos2), calmodulin 1 (phosphorylase kinase delta) (Calm1), E2F transcription factor 1 (E2f1), cyclin-dependent kinase inhibitor 1A (p21, Cip1) (Cdkn1a) and cadherin 12, type 2 (*N*-cadherin 2) (Pcdh7) were increased in the striatum of maneb- and paraquat-treated animals as compared with controls. The expression patterns of Pde1b, E2f1, Eif4g2 and Tubb5 were restored by melatonin, Pdha1, Cdkn1a and Pcdh7 by silymarin and others by both in the striatum of maneb- and paraquat-treated animals (Table 2).

Transcripts associated with growth factor receptors and ion channels

The expression patterns of neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2), insulin-like growth factor 1 (somatomedia C) (Igf1), G protein-coupled receptor 20 (P2ry4), growth factor receptor-bound protein 2 (Grb2), inositol 1,4,5-triphosphate receptor, type 1 (Itpr1), solute carrier family 22 (organic cation transporter), member 2 (Slc22a2) and chloride channel, calcium activated, family

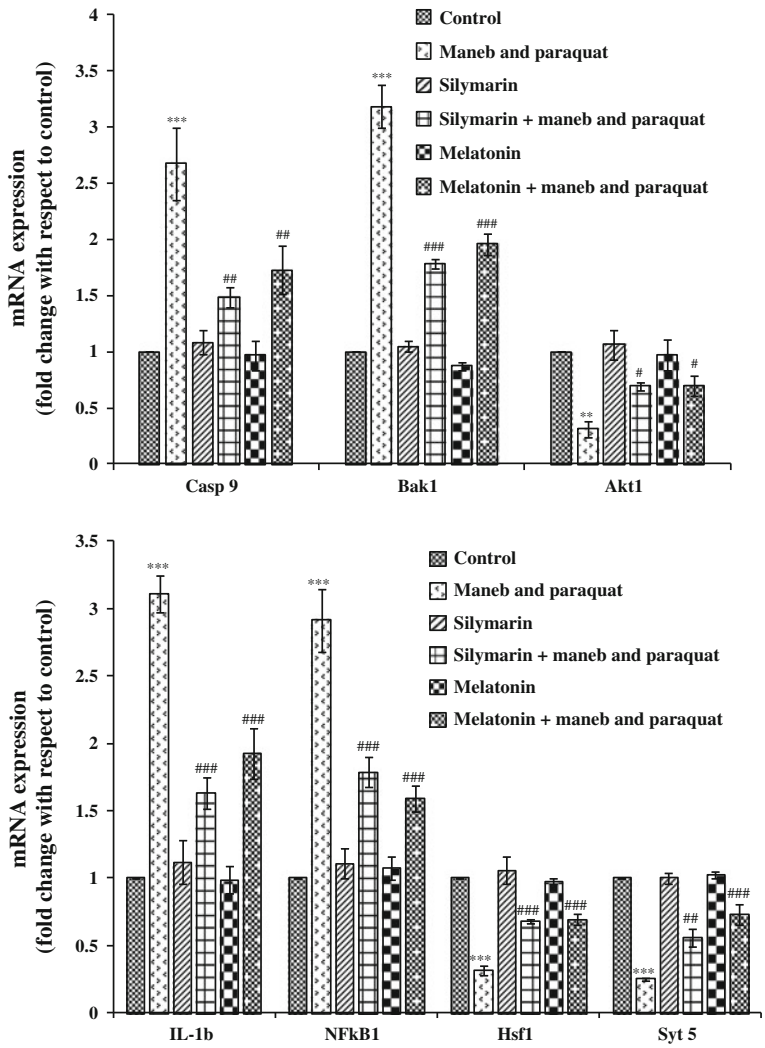

Fig. 1 Bar diagrams showing the differential expression of Casp9, Bak1, Akt1, IL-1β, NFκB1, Hsf1 and Syt5 in the striatum of maneb- and paraquat-treated mice in the presence or absence of silymarin or melatonin. Data were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control values were considered as 1 in all independent sets of experiments; therefore, there is no error bar in controls. The data were calculated by ΔΔCt method and values are expressed in means ± SEM ($n = 3$). The significant changes are expressed as $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with controls and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ as compared with maneb- and paraquat-treated animals

member 1 (Clca1) transcripts were decreased and fibroblast growth factor receptor 3 (achondroplasia) (Fgfr3) and platelet-derived growth factor receptor-alpha (Pdgfra) were increased significantly after maneb- and paraquat-exposure. The expression patterns of Pdgfra, P2ry4, Slc22a2 and Itpr1 were restored by melatonin, Fgfr3 and Clca1 by silymarin and remaining by both in the striatum of maneb- and paraquat-treated animals (Table 2).

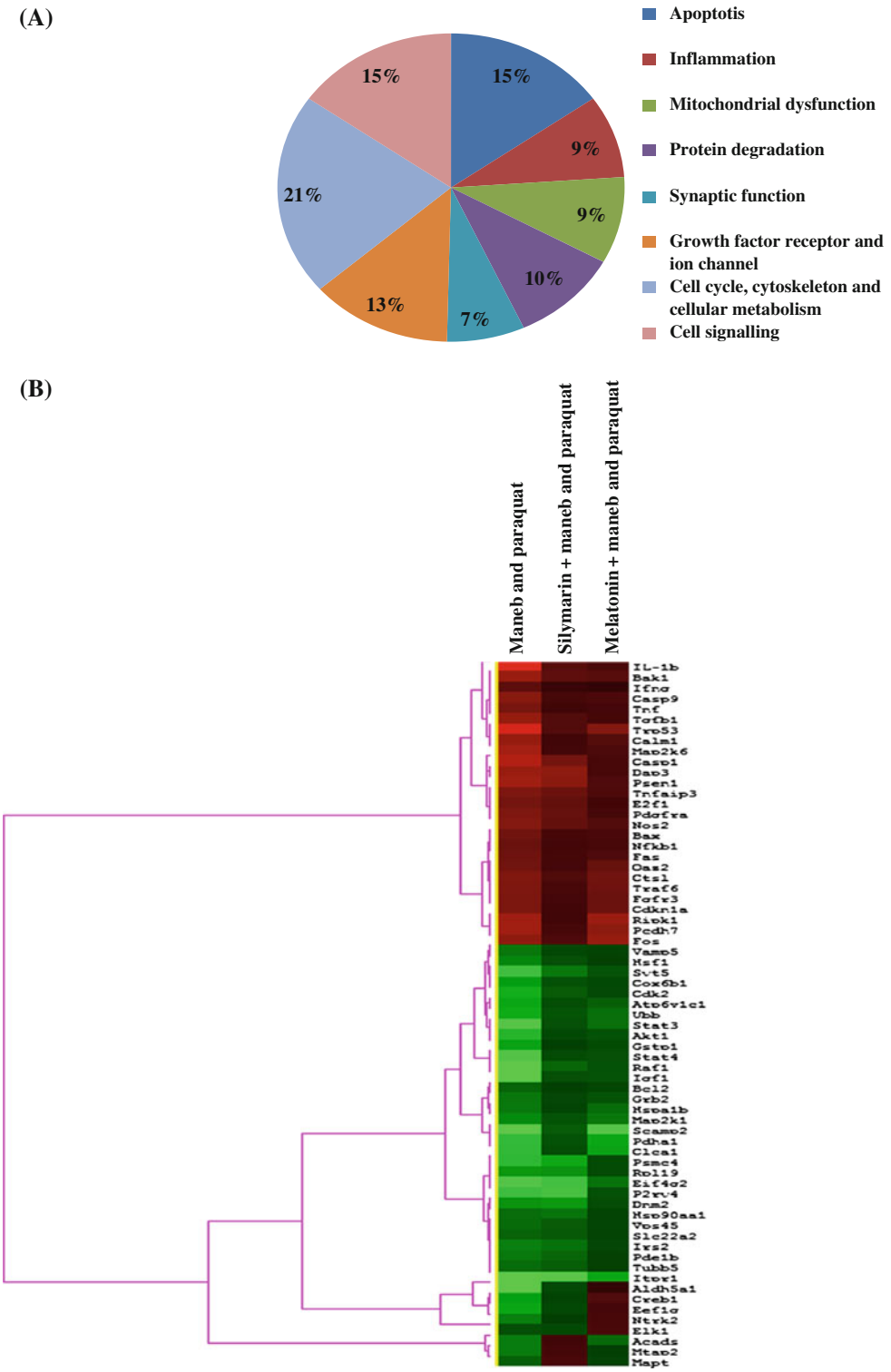

Fig. 2 Pie diagram showing the percentage of the total transcripts associated with various functions (**a**). Cluster display showing the differentially expressed transcripts in the striatum of maneb- and paraquat-treated animals in the presence of silymarin or melatonin (**b**). *Red colour* indicates upregulated while *green colour* indicates downregulated transcripts. (Color figure online)

qRT-PCR

The expression patterns of Casp9, Bak1, Akt1, IL-1β, NFκB1, Hsf1 and Syt5 exhibited the similar trends as observed with microarray experiments (Fig. 1). The expression patterns of Casp9, Bak1, IL-1β and NFκB1 exhibited upregulation while Akt1, Hsf1 and Syt5 exhibited downregulation in maneb- and paraquat-induced PD phenotype. The expression patterns of these transcripts were restored towards normalcy in the animals, which were also treated with silymarin or melatonin.

Gene clustering

All differentially expressed genes were clustered in vein diagram (percent transcripts) and are shown in the form of a tree view (Fig. 2a, b). Maneb- and paraquat-treatment altered the expression of transcripts related to apoptosis (15 % of the total differentially expressed transcripts), inflammation (9 %), mitochondrial function (9 %), protein degradation (10 %), synaptic function (7 %), growth factor receptor and ion channels (13 %), cell cycle, cytoskeleton and cellular metabolism (21 %) and cell signalling (15 %).

Discussion

Maneb- and paraquat-induced Parkinsonism is used as a model since it is one of the most widely studied models and is also environmentally relevant [3]. While silymarin and melatonin are reported to offer neuroprotection, underlying mechanisms are not yet completely deciphered [4, 25, 26]. Discover chips (mouse) microarrays were used in the study since they comprise more than 380 genes in duplicate, which are related to various biological events implicated in the nigrostriatal dopaminergic neurodegeneration, i.e. apoptosis, inflammation, cell signalling, synaptic function, mitochondrial dysfunction, protein degradation, cell cycle, cellular metabolism, cytoskeletal networks, growth factor receptors and ion channels [12, 13]. Since the study assessed the involvements of multiple genes in silymarin- or melatonin-mediated neuroprotection against maneb- and paraquat-induced Parkinsonism, the differential expression of transcripts in maneb- and paraquat-exposed mice was also assessed as compared with controls.

Parkinson's disease is pigeonholed with extrinsic and intrinsic apoptosis and several transcripts, such as Bax, Bcl2, Bcl2-interacting killer (Bik), Bak, bcl-2-interacting mediator of cell death (Bim), casp1, caspase 3, caspase 6, casp8, casp9, etc. are actively involved in disease pathogenesis [14, 27]. Silymarin or melatonin restored the expression patterns of Bak1 and casp9 towards normalcy suggesting that the reduced apoptosis could also lead to neuroprotection [8, 28, 29]. Maneb- and paraquat-induced reduction in Bcl2 [30] and subsequent changes by antioxidants further supports the active role of reduced apoptosis in silymarin- or melatonin-mediated neuroprotection. Similarly, reduced or increased expression of transcripts associated with cell cycle and metabolism indicates that combined exposure to maneb and paraquat impairs cell cycle regulation and cellular metabolism. Melatonin and silymarin co-treatment counteracted maneb- and paraquat-induced modulations towards normalcy showing that antioxidants reduce pesticides-induced impairments in cell cycle and metabolism.

Oxidative stress induces the production of inflammatory cytokines, which in turn induce Fas-mediated apoptosis, nitric oxide synthase expression and microglial activation [3, 6, 7, 31]. Silymarin or melatonin also reduced the microglial activation and expression patterns of inflammatory cytokines, such as IL-1β and NFκB1, which could contribute to neuroprotection against pesticides-induced PD [4, 7]. Since Akt1 is involved in neuronal cell survival [32], an increased expression of Akt1 after silymarin or melatonin treatment could also suggest the neuroprotective role of silymarin or melatonin. Oxidative stress alters the antioxidant enzyme defence system and expression of glutathione-S-transferase (GST), nitric oxide synthase (NOS) and cytochrome P450 (CYP) genes [4, 31, 33, 34]. The levels of GST Pi and NOS2 were altered in maneb- and paraquat-treated mice, which were significantly restored by silymarin or melatonin showing that the selected antioxidants could regulate the expression patterns of antioxidant enzymes and rescue from the increased oxidative stress.

Synaptic transmission is regulated by synaptotagmin and voltage-gated calcium channels [35]. The reduced expression of Syt5 gene, which is involved in synaptic transmission, after pesticides exposure is also in accordance with a previous report [36]. The expression of Syt5 gene was restored by silymarin or melatonin, which supports that silymarin or melatonin rescues from the change in the level of synaptotagmin [37]. Similarly, pesticides altered the expression patterns of Ubb, proteasome subunit, Hsf1, etc. genes while silymarin and melatonin restored the gene expression levels towards normalcy. Such observations suggest that silymarin and melatonin revamp the mitochondrial impairment and proteasomal dysregulation, two major pathways involved in PD pathogenesis [38].

The growth factor receptors such as Ntrk2, fibroblast growth factor receptor, etc. induce several transcription factors that are critical in neuronal survival [13, 39, 40]. Silymarin or melatonin restored the expression levels of growth factor receptors and cytoskeletal genes, which were modulated after maneb- and paraquat-treatment [41], suggesting the involvement of such events in neuroprotection. Dopamine content and microglial activation were also checked employing the standard procedures [4, 5, 42, 43] to indemnify

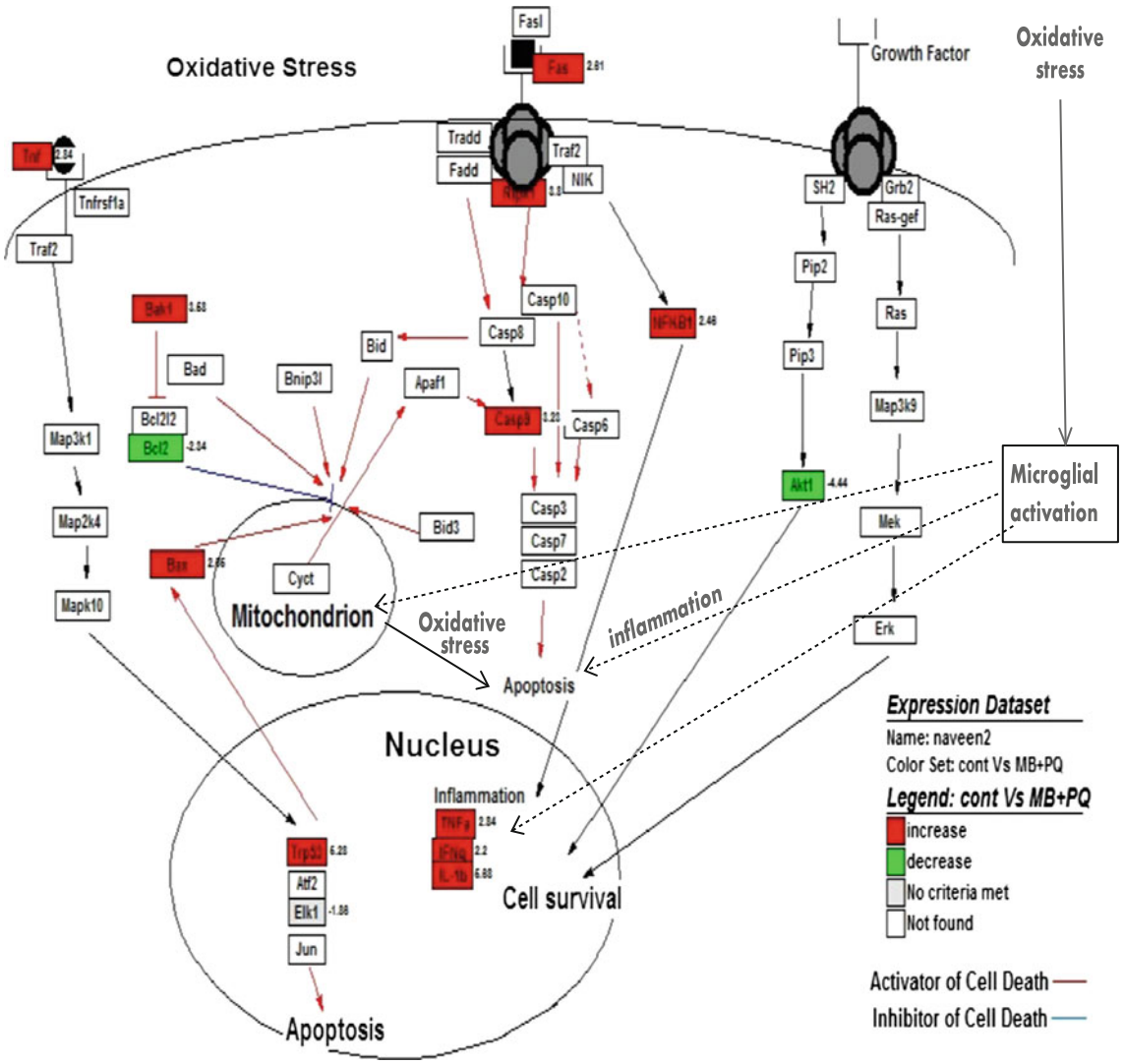

Fig. 3 Putative signalling pathway of silymarin- and melatonin-mediated neuroprotection against maneb- and paraquat-induced nigrostriatal dopaminergic neurodegeneration. *Red colour* indicates upregulation while *green colour* indicates downregulation of the genes. (Color figure online)

that pesticides-treated animals exhibited Parkinsonism and silymarin- and melatonin-treated animals offered neuroprotection [4]. Melatonin is found to synergistically increase the resveratrol- or minocycline-induced neuroprotection [44, 45]. Since silymarin is an antioxidant like resveratrol and is a neuroprotective agent like minocycline, it may be possible that melatonin could synergistically increase the neuroprotective efficacy of silymarin against maneb- and paraquat-induced PD, if administered in combination. However, we did not assess the effect of combined exposure to melatonin and silymarin; therefore, we do not have any experimental evidence to prove the hypothesis.

The maneb- and paraquat-mediated Parkinsonism were improved towards normalcy by silymarin and melatonin showing that they offer neuroprotection by the modulation of multiple pathways, which include oxidative stress, inflammation, apoptosis, mitochondrial dysfunction, etc. (Fig. 3).

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Conflict of interest The authors declare that they have no conflict of interest.

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