Genes critical for development and differentiation of dopaminergic neurons are downregulated in Parkinson's disease

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ABSTRACT

We performed transcriptome analysis using RNAseq on substantia nigra pars compacta (SNpc) from mice after single exposure, 14 days after daily MPTP treatment and Parkinson's disease (PD) patients. Single dose of MPTP resulted in downregulation of genes involved in sodium channel regulation, which was also observed after 14 days. Upregulation of pro-inflammatory pathways was seen after single dose but not after 14 days of MPTP treatment. Dopamine biosynthesis and synaptic vesicle recycling pathways were downregulated both in mice after 14 days of MPTP and PD patients. LMX1B, that is essential for midbrain development and determination of dopaminergic phenotype and other genes including FOXA1, RSPO2, KLHL1, EBF3, PITX3, RGS4, ALDH1A1, RET, FOXA2, EN1, DLK1, GFRA1, LMX1A, NR4A2, GAP43, SNCA, PBX1 and GRB10 were downregulated in human PD. Downregulation of ensemble of genes involved in development and differentiation of dopaminergic neurons indicate their critical involvement in pathogenesis and progression of human PD.

Introduction

Parkinson's disease (PD) is a debilitating neurodegenerative movement disorder. The cardinal clinical symptoms of Parkinson's disease include rigidity, bradykinesia and resting tremors. PD is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). While it has been more than 200 years since PD was first characterized by James Parkinson[1] , lack of thorough understanding of the mechanisms that underlie disease pathogenesis has been a major obstacle in discovery of curative treatment modalities. Levodopa administration has been the mainstay for symptomatic treatment for almost sixty years[2,3] ,we do not, as yet, have a disease modifying therapy that can slow down disease progression.

Familial PD is seen in less than 10% of cases, the majority are sporadic[4] . The etiology of both familial and sporadic PD is not yet clearly understood, and it is considered to be a complex, multifactorial disorder. Several risk factors have been implicated including oxidative stress, mitochondrial[5,6] and proteasomal dysfunction[7,8] , dopamine metabolism[9] and iron accumulation[10,11] . However, the exact cause underlying PD pathogenesis remain poorly understood. Therefore, understanding the mechanisms that lead to neurodegeneration of dopaminergic neurons in SNpc is vital.

Transcriptomics allows us to identify dynamic changes in gene expression between different physiological states. Global gene expression analysis using microarray has been deployed to identify critical changes in gene expression in PD[12] . Many pathways, such as mitochondrial electron transport system, ubiquitin-proteasome system, apoptosis, actin cytoskeleton, inflammation and synaptic transmission have been reported to be dysregulated in PD studies[13,14] . However, microarray lacks the sensitivity

to detect genes which are expressed in low levels, that is they have limited dynamic range of detection[15,16] . Moreover, there is low signal to noise ratio as well as there is an upper limit to detection of transcripts beyond which the signal gets saturated. More recently approaches using RNA sequencing (RNA-seq) offer good alternative to examine global gene expression changes in disease state.

We, therefore used RNA seq to identify global gene expression changes in mouse model of PD following single and chronic exposure to MPTP. Further, RNA-seq was performed to study gene expression alterations in SNpc from human PD patients compared with age-matched controls.

Methods

Animals and MPTP dosing C57BL/6J male mice (3-4 months; 25-30 grams), procured from Central animal facility of Indian Institute of Science were used for all animal experiments. Institutional guidelines for the use and care of animals were followed. Animals were housed in groups of five and had access to pelleted diet and water, ad libitum. For 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) mouse model of dopaminergic loss[17] , MPTP (Sigma-Aldrich Cat# M0896) dissolved in normal saline was given at a dose of 30mg/kg body weight sub-cutaneously to mice as a single injection or daily for 14 days. MPTP injections were carried out in an isolated clean air room in the Central Animal Facility, Indian Institute of Science, Bangalore. Adequate safety precautions were taken for proper handling of MPTP during preparation and injection and for disposal of materials and samples contaminated with MPTP. The controls were injected with an equivalent volume of normal saline. Animals were sacrificed 24 hours after the last MPTP dose.

Animal experiments were approved by the institutional animal ethical review board, named 'Institutional Animal and Ethics Committee' of Indian Institute of Science (Protocol# CAF/Ethics/267/2012). Animals were handled according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Mouse brain SNpc dissection MPTP treated mice were decapitated following cervical dislocation post 24 hours of the last dose of MPTP in the treatment paradigm following cervical dislocation. Mouse brain was placed on mouse brain matrix (Ted Pella Inc. Cat# 15050) and 1mm thick slices of the brain were obtained for the dissection of SNpc, under cold and sterile conditions. Then, SNpc was dissected out from these slices under stereomicroscope using anatomical markers.

Human brain tissue dissection All experiments on human autopsy brain tissue were carried out following approval from Institutional Human Ethics Committee of the Indian Institute of Science and all guidelines were followed (IHEC approval no. 10/1/2015).

For the dissection of SNpc, slides containing sections of human autopsy midbrain tissue from PD patients and age-matched controls were obtained from the ICM Brain Bank and the Neuroceb Pitié-Salpêtrière hostital, Paris. Control brains were obtained from individuals without neurologic or psychiatric disorders. Patients with PD displayed the characteristics of the disease including akinesia, rigidity and/or resting tremor. All patients with PD were treated with dopaminergic agonists and/or L-DOPA and displayed or had displayed dyskinesia. The clinical diagnosis was confirmed by neuropathological examination including the presence of Lewy bodies at least in the substantia nigra. Mean post-mortem delay and age at death did not differ between control subjects and patients with PD. A table with details on patients has been appended in supplementary

(Supplementary table S1). SNpc was scraped off the surface of the slides, kept on dry ice under sterile conditions using anatomical landmarks.

RNA isolation All the reagents used for RNA isolation and cDNA synthesis were prepared in RNAase free conditions using DEPC water. RNA from mouse brain tissue was isolated using TRIzol reagent (Invitrogen Cat# 15596018) and bromo-chlorophenol (BCP) (Molecular Research Centre, Inc. Cat# BP151)[18] . RNA was isolated from human autopsy brain tissue (obtained from the Paris Brain Banks) using the RNeasy Plus Universal Mini Kit (Qiagen Cat# 73404).

RNA quality check, cDNA library preparation and RNA sequencing Integrity and purity of RNA samples was determined using Nanodrop, Qubit and BioAnalyzer and samples with Rin value less than 5 were not used. The NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB# E7420G, 30061409) was used for preparing the strand specific libraries from mouse RNA. The sequencing was performed in paired end manner, generating 2X 75 bp long reads and a total of 44-50 million reads. RNA-seq library preparation for human tissue samples was performed using the TruSeq RNA Library Prep Kit v2 (Catalog IDs: RS-122-2001, RS-122-2002). These libraries were prepared in an unstranded manner. The sequencing was performed in paired end manner, generating 2X 100 bp long reads and a total of about 40 million reads. While RNA sequencing on mouse SNpc libraries was performed on Illumina HiSeq2000, RNA-seq on human SNpc libraries was performed on Illumina HiSeq2500.

Differential gene expression and pathway analysis Analysis of RNA-seq data was performed on Linux operating system Debian GNU/Linux 8 (Jessie). The raw sequencing data, in the form of FastQ files was checked for quality using FastQC, following which the low quality reads and adapter sequences were removed using

Trimmomatic (v0.36)[19] . Further, TopHat2 (v2.1.1)[20] was used for alignment of reads to mouse or human genomes. Ensembl genome GRCm38 was used as mouse genome reference genome and GRCh37 was used as human reference genome. The resultant alignment files are in the bam (binary alignment/map) format, which were further sorted in the order of names of genes using Samtools, 0.1.19[21] and finally the numbers of counts for each read in each sample were generated using htseq-count[22] . DESeq2 version 1.22.2[23] was used for normalization of read counts. DESeq2 analysis was performed on R version 3.5.1.

Normalization by Cufflinks methods[24] was repeated on the human data after alignment by following the Cufflinks (v2.2.1): Cuffmerge-Cuffdiff pipeline and differentially expressed genes were identified for validation of DESeq2 data. Data on differential gene expression of developmental genes was also validated by the changing the DESeq2 linear model to account for age, post-mortem interval (PMI) and RIN. The altered model was: age+PMI+RIN+condition.

All subsequent data analysis was performed on genes that were differentially expressed and had adjusted p values less than 0.05 as determined using DESeq2 normalization.

Functional annotation clusters from differentially expressed genes that were significantly different were created using DAVID bioinformatics resources 6.8[25,26] and GOplot 1.0.2[27] was used for visualization of the data. pheatmap 1.0.12[28] was used for generation of heatmaps and volcano plots were created using EnhancedVolcano 1.5.0[29] .
Protein-protein associations that may result from co-differential expression of genes were drawn using String database[30] . The Venn diagrams were plotted using the tool available at the url: http://bioinformatics.psb.ugent.be/webtools/Venn/.

Quantitative real time PCR Human total RNA (200ng) was used for first strand cDNA synthesis using random hexamers, dNTPs and reverse transcriptase from the high capacity cDNA reverse transcription kit (Applied Biosystems Cat# 4368814). Quantitative real time PCR (qRT-PCR) was performed using SYBR green chemistry for GAP43, RET, LMX1B, EN1 and DAT. The nucleotide sequences for primers used for human tissue gene expression analysis and the qRT-PCR conditions are provided in supplementary tables S2 and S4. Two endogenous controls, namely β-actin and GAPDH were used for normalization. For mouse SNpc samples, qRT-PCR was performed using SYBR green chemistry for Dat, Th, Lmx1b, En1, Ret and Gap43. The nucleotide sequences for primers used for mouse tissue gene expression analysis and the qRT-PCR conditions are provided in supplementary table S3. 18S rRNA was used for normalization.

LMX1B over expression construct Expression construct for human LMX1B was subcloned from the tetO-ALN plasmid[31] . tetO-ALN was a gift from John Gearhart (Addgene plasmid# 43918; `http://n2t.net/addgene:43918`; RRID: Addgene_43918). The open reading frame (ORF) for human LMX1B was PCR-amplified using the following primer sequences: Forward primer: TAAGCATCTAGAATGTTGGACGGCATCAAG; Reverse primer: TGCTTAGGATCCTCAGGAGGCGAAGTAGGAACT. The ORF thus PCR-amplified was cloned into the pcDNA™3.1/V5-His TOPO™ TA vector (ThermoFisher Scientific, Cat# K480040) following manufacturer's protocol.

Experiments with SH-SY5Y cells SH-SY5Y cells (ATCC® CRL-2266™) cultured in Minimum Essential Medium Eagle (MEM; Sigma, Cat# M0643) that was supplemented with 10% v/v fetal bovine serum (FBS; Gibco, Cat# 26140079, Origin: U.S) and 1X

penicillin-streptomycin (Gibco, Cat# 15070063). SH-SY5Y cells were plated at a confluency of 70% on glass cover slips (VWR, Cat# 26022) that were acid washed and precoated with poly-D-lysine (0.1 mg/ml) in 12-well plates.

Within 16 hours of plating, SH-SY5Y cells were transfected with pcDNA3.1-LMX1B using Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen, Cat# 15338030) in OptiMEM medium (Gibco, Cat# 22600-050). The media was changed to MEM with FBS and penicillin-streptavidin after 6 hours.

SH-SY5Y cells were treated with MPP^+ (1mM; Sigma-Aldrich D048) 48 hours after transfection with pcDNA3.1-LMX1B. The cells were washed with phosphate buffered saline (PBS) and fixed with 2% paraformaldehyde (w/v) 24 hours after MPP^+ exposure.

The extent of cell death following MPP^+ treatment with and without LMX1B overexpression was assessed by using the terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end-labeling (TUNEL) method (In situ cell death detection kit, TMR red, Roche, Cat# 12156792910), according to the manufacturer's protocol. Ten fields were imaged from three independent coverslips for each experimental condition. Analysis was performed to quantify the average number of TUNEL-positive (Red) cells and was blinded.

Statistics Samples were analyzed in triplicates. Relative gene expression was analyzed using $\Delta\Delta$Ct method. Data was analyzed and represented using Graphpad Prism (Graphpad Prism Inc, USA). Statistical significance was determined using Student's *t* test. All data are presented as mean $\pm$ SEM.

Results

Gene expression changes in mouse model of PD using MPTP RNA sequencing was performed on SNpc, dissected (Fig. 1A) from mice treated with a single dose of MPTP (30mg/kg body weight) or daily for 14 days. While single dose of MPTP represents the first hit without substantial cell loss; about 50% loss of dopaminergic neurons is seen after 14 days of MPTP treatment[32]. Indeed, down-regulation of several dopaminergic genes such as, dopamine transporter (Dat), DOPA decarboxylase (Ddc) and vesicular monoamine transporter 2 (Vmat2) was observed after 14 days of MPTP treatment (Fig. 1B) but not after single exposure. Only Th and Pitx3 were downregulated after 1 and 14 days of MPTP treatment.

Differential gene expression analysis of RNA-seq data from mouse SNpc after 24 hours following single exposure to MPTP revealed significantly (adjusted $p < 0.05$) altered expression of 472 genes, 291 of which were up-regulated and 181 were down-regulated (Fig. 1C and 1E). However, analysis of data from mouse SNpc after 14 days of MPTP treatment showed significant (adjusted $p < 0.05$) altered expression of 74 genes, 23 of which were up-regulated and 51 were down-regulated (Fig. 1D and 1F). Thus, fewer genes were perturbed as the disease progressed and cell loss continued. As evident from the Venn diagram (Fig. 1G), there was little overlap between the two sets suggesting the involvement of different molecular pathways during early and later stages of MPTP treatment. Further, there was greater perturbation in terms of number of differentially expressed 24 hours after single dose of MPTP, which could be due to the adaptive response to the toxin.

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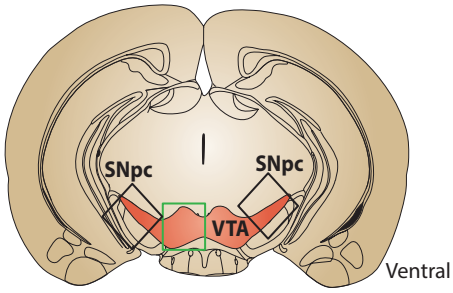

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Symbol	Gene names	Log_2 Fold Change	p(adj)
Th	tyrosine hydroxylase	-1.19	1.33E-03
Slc6a3 (DAT)	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	-1.14	2.21E-04
Ddc	DOPA decarboxylase	-0.96	1.63E-03
Slc18a2 (VMAT2)	solute carrier family 18 (vesicular monoamine), member 2	-0.93	3.62E-03

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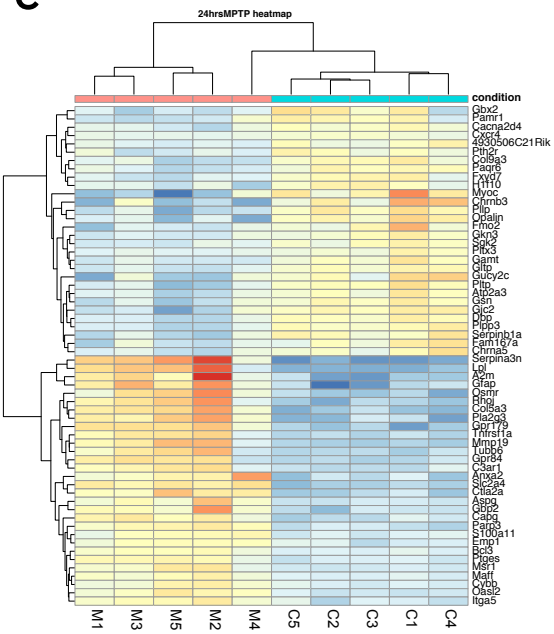

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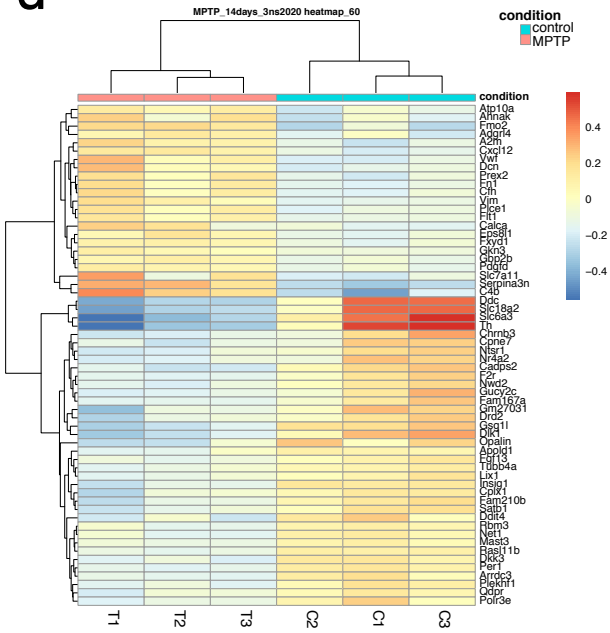

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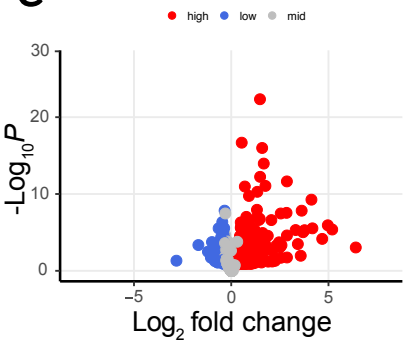

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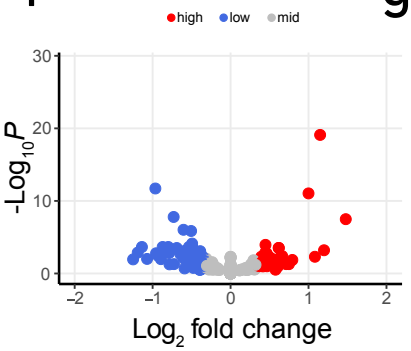

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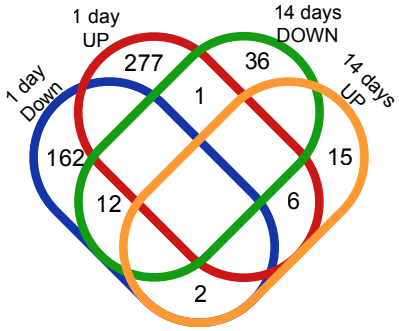
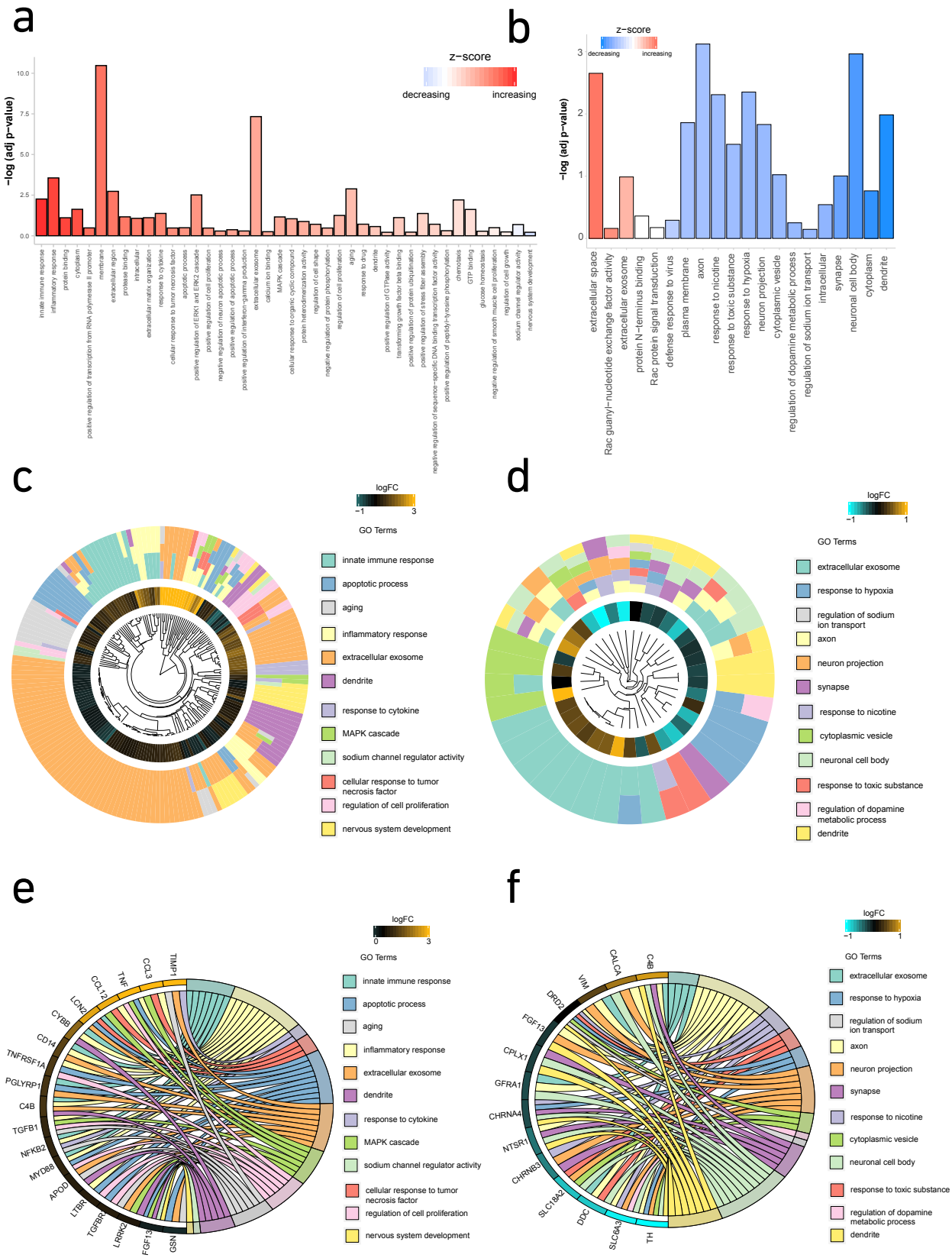


Figure 1: **Differential gene expression in SNpc from MPTP mouse model of PD. (a) A diagrammatic representation of SNpc dissection using coronal mouse midbrain sections. (b) Table depicting downregulation of genes specific to dopaminergic neurons in SNpc from 14 days MPTP treated mice. The top 60 significantly differentially expressed genes after 1 day (c) and 14 days (d) of MPTP treatment, represented through heatmaps along with their $\log_2$ fold change. (e) and (f) Volcano plots for differentially expressed genes in SNpc from 1 day and 14 days MPTP treated mice SNpc, respectively. (g) Venn diagram showing the overlap of the upregulated and downregulated genes between 1 day and 14 days MPTP treatment.**

Perturbation of different pathways seen at early and late stage of MPTP mouse model of PD Identification of gene ontology (GO) pathways that were populated by genes that were significantly up or downregulated in SNpc after single exposure to MPTP revealed an upregulation of pathways, such as innate immune response, inflammatory response, response to cytokine, cellular response to tumor necrosis factor, apoptotic processes, extracellular exosome, MAPK cascade, regulation of cell proliferation, aging and dendrite and a downregulation of pathways, such as nervous system development and sodium channel regulator activity (Fig. 2A). A similar analysis for the RNA-seq data from 14 days MPTP mouse SNpc did not show perturbation of innate immune response or inflammatory response. However, upregulation of pathways involved in functioning of plasma membrane, extracellular space, extracellular exosome and downregulation of pathways involved in functioning of axon, response to nicotine, response to toxic substances, response to hypoxia, neuron projection, cytoplasmic vesicle, regulation of dopamine metabolic process, regulation of sodium ion transport, synapse, neuronal cell body and dendrite (Fig. 2B).

The circular plots for single dose (Fig. 2C) and 14 days (Fig. 2D) of MPTP treatment shows hierarchical clustering of the significant genes based on functional categories and how they populate these categories along with their $\log_2$ fold change. Further, chord diagrams for differentially expressed pathways and their resident genes after single dose (Fig. 2E) and 14 days (Fig. 2F) of MPTP treatment show the significantly differentially expressed genes that populate at least three different pathways and their $\log_2$ fold change.

The early stage of MPTP mouse model thus presents a canvas of molecular pathways that is quite dissimilar to what is observed at late stage of the model. It is also intriguing to note the absence of differentially expressed genes related to innate immunity and inflammatory response in SNpc from mouse treated with MPTP for 14 days, wherein about 50% of dopaminergic neurons are lost.


a
b
z−score
decreasing
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innate immune response
inflammatory response
protein binding
cytoplasm
positive regulation of transcription from RNA polymerase II promoter
membrane
extracellular region
protease binding
intracellular
extracellular matrix organization
response to cytokine
cellular response to tumor necrosis factor
apoptotic process
positive regulation of ERK1 and ERK2 cascade
positive regulation of cell proliferation
negative regulation of neuron apoptotic process
positive regulation of apoptotic process
positive regulation of interferon−gamma production
extracellular exosome
calcium ion binding
MAPK cascade
cellular response to organic cyclic compound
protein heterodimerization activity
regulation of cell shape
negative regulation of protein phosphorylation
regulation of cell proliferation
aging
response to drug
dendrite
positive regulation of GTPase activity
transforming growth factor beta binding
positive regulation of protein ubiquitination
positive regulation of stress fiber assembly
negative regulation of sequence−specific DNA binding transcription factor activity
positive regulation of peptidyl−tyrosine phosphorylation
chemotaxis
GTP binding
glucose homeostasis
negative regulation of smooth muscle cell proliferation
regulation of cell growth
sodium channel regulator activity
nervous system development
extracellular space
Rac guanyl−nucleotide exchange factor activity
extracellular exosome
protein N−terminus binding
Rac protein signal transduction
defense response to virus
plasma membrane
axon
response to nicotine
response to toxic substance
response to hypoxia
neuron projection
cytoplasmic vesicle
regulation of dopamine metabolic process
regulation of sodium ion transport
intracellular
synapse
neuronal cell body
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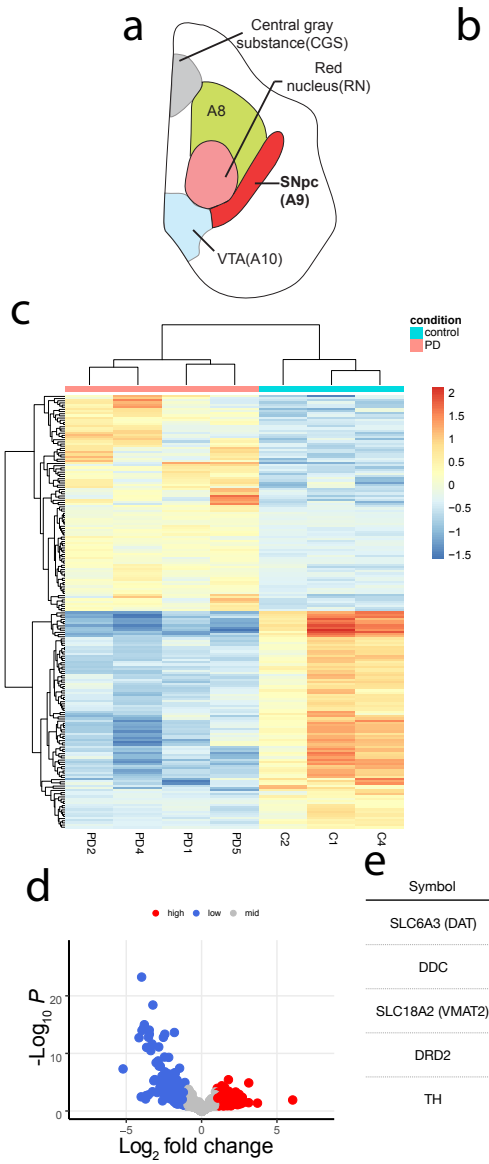
TNFRSF1A
PGLYRP1
C4B
TGFB1
NFKB2
MYD88
APOD
LTBR
TGFBR3
LRRK2
FGF13
GSN
CALCA
C4B
VIM
DRD2
FGF13
CPLX1
GFRA1
CHRNA4
NTSR1
CHRNB3
SLC18A2
DDC
SLC6A3
TH

Figure 2: **Differentially expressed pathways enriched in SNpc from MPTP mouse model of PD. (a) and (b) Bar diagrams for functional clusters derived using DAVID for significantly differentially expressed genes in 1 day and 14 days MPTP mouse model of PD, respectively. Significantly differentially expressed genes and their distribution into selected pathways hierarchically clustered according to functional categories along with the adjusted p values for 1 day (c) and 14 days (d) MPTP treated mice SNpc using circular plots. The innermost space represents the hierarchical clustering of the genes while the inner circle represent the $\log_2$ fold change. (e) and (f) Chord diagrams for selected pathways depicting the significant genes that belong to at least three different pathways with their $\log_2$ fold change for 1 day and 14 days MPTP mouse model of PD, respectively.**

Gene expression changes in SNpc from human PD The anatomical landmarks used for dissection of SNpc are depicted in Fig. 3A. The top 30 differentially expressed genes are tabulated in Fig. 3B. Differential gene expression analysis through DESeq2 revealed significantly (adjusted $p < 0.05$) altered expression of 367 genes, of which 103 were up-regulated and 264 were down-regulated in SNpc of human PD brain (Fig. 3C and 3D). Markers of dopaminergic neuron phenotype such as DAT, VMAT2, DDC, DRD2 and TH were significantly downregulated (Fig. 3E), which served as a positive indication of degeneration of dopaminergic neurons in SNpc in these samples. Components of the ubiquitin-proteasome pathway such as, PSMD12, UBE2T, UBFD1 and UCHL1 were down-regulated in the human PD. Of the genes that were down-regulated, UCHL1 (also known as PARK5) has been associated with familial PD[33]. Dysregulation of the ubiquitin-proteasome pathway is one of the well-studied mechanisms implicated in the pathogenesis of PD[34].

Neuron specific genes such as MAP2, RBFOX3 and TUBB3 were unchanged. Further, the mRNA expression of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as assessed using qRT-PCR, remained unchanged as seen after normalization with ribosomal protein L13 (RPL13) in human PD (Supplementary Fig 1).

Pathway analysis reveals down regulation of dopaminergic neuron differentiation in human PD Pathway analysis was performed using the significantly differentially expressed genes showed downregulation of functional clusters, such as those involved in oxidation-reduction process, neuron projection, synaptic vesicle recycling, regulation of synaptic plasticity, dopamine biosynthetic process, dopaminergic neuron differentiation and locomotory behavior (Fig. 4A). We also clustered significantly expressed genes according to a few selected functional categories (Fig. 4B). Further, the chord diagram shows distribution of differentially expressed genes in selected pathways with their adjusted p values (Fig. 4C). An interesting set of genes that were perturbed significantly as highlighted is of those that belong to dopaminergic neuron differentiation as also those that define midbrain dopaminergic neuron development and maintenance. These include FOXA1, RSPO2, KLHL1, LMX1B, EBF3, PITX3, RGS4, ALDH1A1, RET, FOXA2, EN1, DLK1, GFRA1, LMX1A, NR4A2, GAP43, SNCA, PBX1 and GRB10 (Fig. 4D).



b

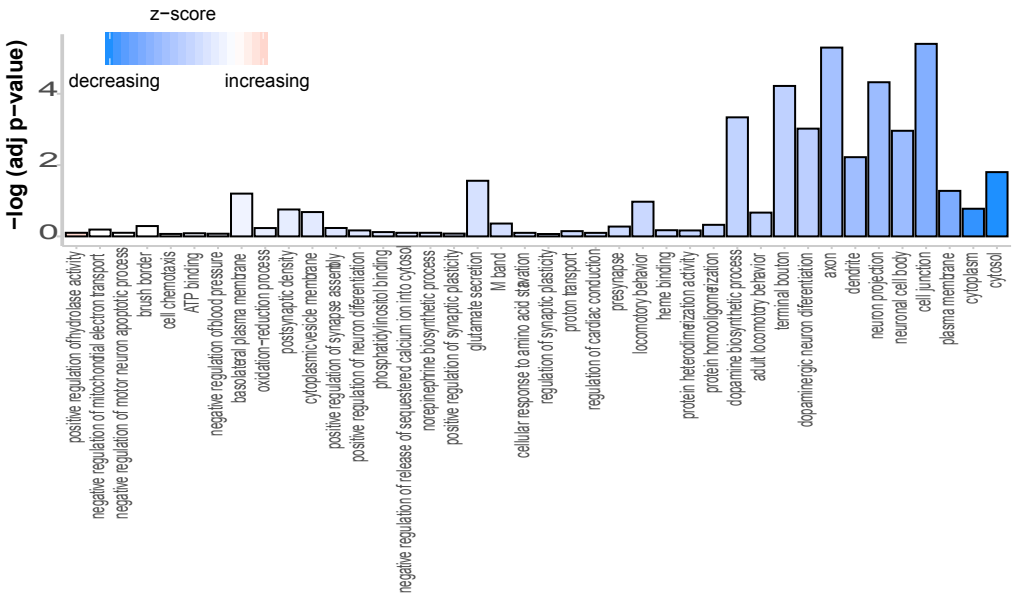
Symbol	Gene names	Log2 Fold Change	p(adj)
FABP1	fatty acid binding protein 1	3.71	4.29E-02
MYO1A	myosin IA	3.13	1.32E-05
POU4F2	POU class 4 homeobox 2	3.12	3.61E-02
CRLF1	cytokine receptor like factor 1	2.70	4.28E-03
NUTM2A	NUT family member 2A	2.49	1.31E-03
C1orf87	chromosome 1 open reading frame 87	2.26	5.03E-04
LINC00702	long intergenic non-protein coding RNA 702	2.17	1.2E-02
CCDC170	coiled-coil domain containing 170	1.98	2.81E-02
CYP4F3	cytochrome P450 family 4 subfamily F member 3	1.90	1.92E-02
THNSL2	threonine synthase like 2	1.90	4.49E-03
PRLHR	prolactin releasing hormone receptor	1.81	3.87E-04
LINC01549	long intergenic non-protein coding RNA 1549	1.78	3.72E-06
TMEM30B	transmembrane protein 30B	1.74	1.58E-02
FAM131C	family with sequence similarity 131 member C	1.63	1.12E-03
MEGF6	multiple EGF like domains 6	1.60	1.95E-02
FST	follistatin	-3.25	4.49E-04
KLHL1	kelch like family member 1	-3.29	2.29E-12
KCNJ6	potassium voltage-gated channel subfamily J member 6	-3.37	3.25E-11
DDC	dopa decarboxylase	-3.47	4.61E-14
RSPO2	R-spondin 2	-3.47	8.83E-15
FOXA1	forkhead box A1	-3.53	1.55E-13
ZAR1	zygote arrest 1	-3.53	1.57E-03
NTSR1	neurotensin receptor 1	-3.65	9.01E-12
ELOVL3	ELOVL fatty acid elongase 3	-3.68	2.13E-03
HBG2	hemoglobin subunit gamma 2	-3.72	8.11E-03
SLC10A4	solute carrier family 10 member 4	-3.80	1.03E-15
SLC6A3	solute carrier family 6 member 3	-3.85	3.87E-15
AGTR1	angiotensin II receptor type 1	-3.96	1.01E-14
SDC1	syndecan 1	-4.15	1.88E-13
SLC35D3	solute carrier family 35 member D3	-5.21	4.91E-08

e

Symbol	Gene names	Log2 Fold Change	p(adj)
SLC6A3 (DAT)	solute carrier family 6 member 3	-3.85	3.87E-15
DDC	dopa decarboxylase	-3.47	4.61E-14
SLC18A2 (VMAT2)	solute carrier family 18 member A2	-3.23	3.91E-19
DRD2	dopamine receptor D2	-2.88	2.02E-09
TH	tyrosine hydroxylase	-2.37	7.07E-05

Figure 3: **Differential gene expression in SNpc from human autopsy tissue samples.(a) Graphical representation of human midbrain nuclei, viz. central gray substance (CGS), red nucleus (RN), A8, substantia nigra pars compacta (SNpc), ventral tegmental area (VTA). (b) Differential gene expression in PD-from human autopsy tissue. The list displays the top 30 upregulated and downregulated genes in SNpc from PD autopsy tissue. The $\log_2$ fold change and adjusted p value are also provided along with gene names. (c) Heatmap depicting differentially expressed genes in SNpc from human PD (d) Volcano plot representing the relative numbers of upregulated and downregulated genes. (e) Down-regulation of dopaminergic neuron markers in SNpc from autopsy tissue from PD patients.**

a



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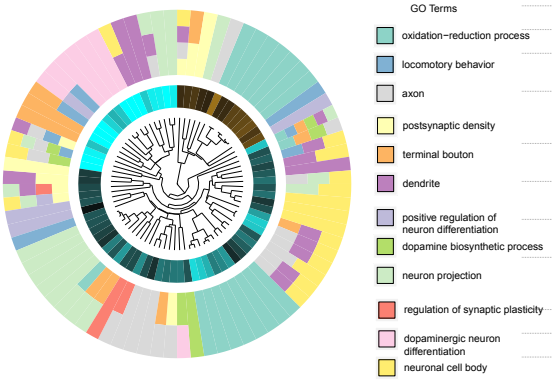

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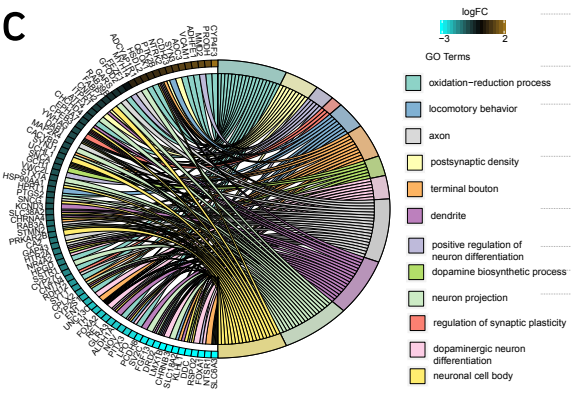

d

Symbol	Gene names	Log2 Fold Change	p value	p(adj)
FOXA1	forkhead box A1	-3.53	8.24E-17	1.55E-13
RSPO2	R-spondin 2	-3.47	2.34E-18	8.84E-15
KLHL1	kelch like family member 1	-3.29	1.58105E-15	2.29216E-12
LMX1B	LIM homeobox transcription factor 1 beta	-3.11	6.38E-09	3.54E-06
EBF3	EBF transcription factor 3	-2.61	5.71E-09	3.26E-06
PITX3	paired like homeodomain 3	-2.53	5.48E-06	1.1E-03
RGS4	regulator of G protein signaling 4	-2.46	2.9E-08	1.3E-05
ALDH1A1	aldehyde dehydrogenase 1 family member A1	-2.44	2.03E-17	4.61E-14
RET	ret proto-oncogene	-2.40	7.81274E-09	4.09018E-06
FOXA2	forkhead box A2	-2.38	2.25E-05	3.34E-03
EN1	engrailed homeobox 1	-2.17	5.51E-10	3.71E-07
DLK1	delta like non-canonical Notch ligand 1	-2.14	5.41E-05	6.49E-03
GFRA1	GDNF family receptor alpha 1	-1.69	9.63E-07	2.49E-04
LMX1A	LIM homeobox transcription factor 1 alpha	-1.65	1.05E-03	5.23E-02
NR4A2	nuclear receptor subfamily 4 group A member 2	-1.52	2.7042E-05	3.83E-03
GAP43	growth associated protein 43	-1.50	1.22E-06	3.06E-04
SNCA	synuclein alpha	-1.04	1.31E-05	2.11E-03
PBX1	PBX homeobox 1	-0.28	3.39E-02	3.22E-01
GRB10	growth factor receptor bound protein 10	-0.25	4.11E-02	3.51E-01

Figure 4: **Differentially expressed pathways enriched in SNpc from human autopsy tissue samples. (a) Bar diagram of the differentially regulated functional clusters as derived using DAVID from the significantly differentially expressed genes. (b) Circular plot for the significant genes enriched in selected pathways where the innermost space represents the hierarchical clustering of the genes while the inner circle represent the $\log_2$ fold change. (c) Significantly differentially expressed genes that belong to at least two selected functional clusters and their $\log_2$ fold change as represented using a chord diagram. (d) Table of genes that are involved in development, differentiation and maintenance of midbrain dopaminergic neurons with their $\log_2$ fold change, p value and adjusted p value.**

We validated the downregulation of the genes belonging to the pathway related to differentiation of midbrain dopaminergic neurons, viz. GAP43, RET, LMX1B and EN1 in SNpc from PD autopsy tissue using qRT-PCR. Down-regulation of DAT, a marker for dopaminergic neurons was seen (Fig. 5A), which is expected following dopaminergic neuronal death in SNpc. This is indeed an interesting observation considering that genes involved in differentiation of dopaminergic phenotype are indeed important for its maintenance and their downregulation could impact the health of dopaminergic neuron.

We further validated the down-regulation of genes using an alternate method of normalization, that is, Cufflinks and also after regressing the data for age, post mortem interval (PMI) and RIN using DESeq2 (Supplementary tables 3 and 4) and found that majority of the selected genes were significantly downregulated through both the analyses. Though the downregulation of GAP43 does not have a significant adjusted p value but only a significant p value, it was significantly down-regulated in the qRT-PCR experiments.

The RNA-seq data from mouse SNpc following MPTP treatment for 14 days did not show significant adjusted p values for Lmx1b, En1 and Ret even though the raw reads had decreased by 39, 18 and 37%, respectively. However, these genes were significantly downregulated when examined using qRT-PCR (Fig. 5B). The downregulation of these genes in mouse model of PD demonstrate that the dysregulation of this pathway occurs both in mouse and human.

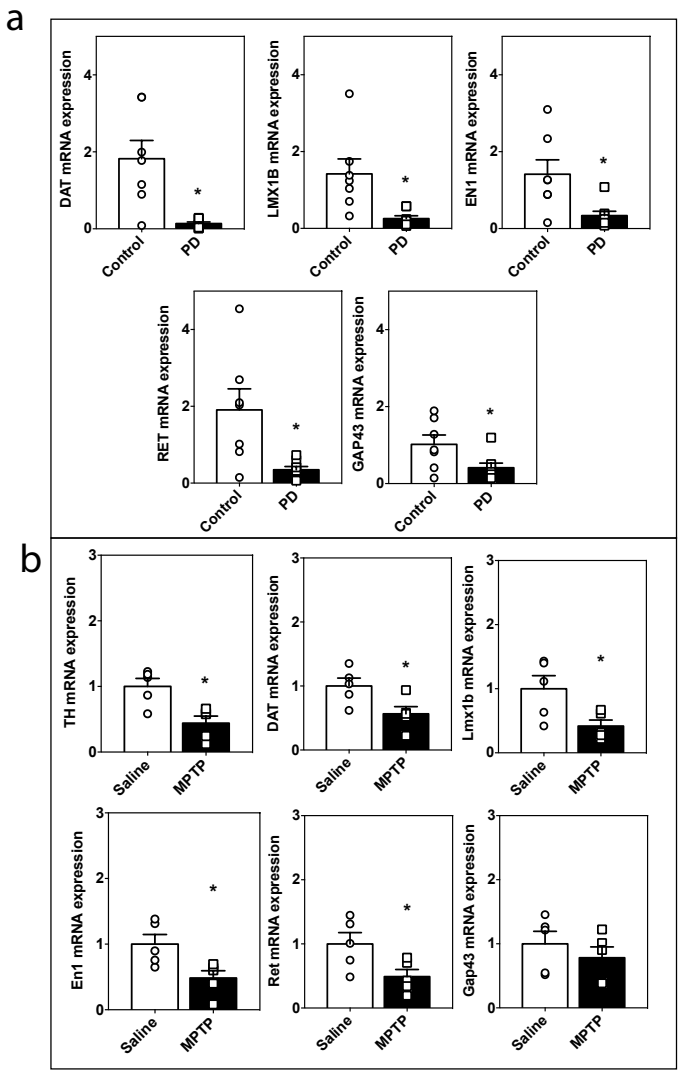

Figure 5: **Validation of down-regulation of genes involved in development and differentiation of midbrain DA neurons through qRT-PCR.(a) Relative level of gene expression of DAT, LMX1B, EN1, RET and GAP43 in on human autopsy SNpc as compared to age-matched controls as quantified using qRT-PCR. (b) Significant downregulation of TH, Dat, Lmx1b, En1 and Ret but not of Gap43 observed in SNpc from C57BL/6 mice treated with MPTP (s.c.) for 14 days. Each dot represents an individual on the graphs. Comparisons between the groups were done using unpaired Student's t test. n=5-8; data has been presented as mean ± SEM. indicates $p<0.05$.**

Protein-protein interactions that can potentially result from co-expression changes in the genes involved dopaminergic neurons differentiation, is depicted in Fig. 6A using String database[30] . Further, mapping these interactions based on existing data (Fig. 6B) revealed LMX1B as an important gene upstream to the expression of many genes involved in dopaminergic midbrain neuron development, differentiation and maintenance. Thus, we see that the whole ensemble of genes (LMX1B, FOXA1, RSPO2, KLHL1, LMX1B, EBF3, PITX3, RGS4, ALDH1A1, RET, FOXA2, EN1, DLK1, GFRA1, LMX1A, NR4A2, GAP43, SNCA, PBX1 and GRB10) that are essential for dopaminergic neuron development, differentiation and maintenance are downregulated in human PD, including those that are downstream of LMX1B. Therefore, we overexpressed LMX1B and assessed its effect on MPP^+ mediated cell death.

Lmx1B overexpression rescues SHSY-5Y from MPP^+ toxicity Recombinant human LMX1B was overexpressed in SH-SY5Y neuronal cell line, which were then exposed to parkinsonism-inducing toxin, MPP^+. We observed that while exposure to MPP^+ led to significant death in SH-SY5Y cells as measured using TUNEL assay, overexpression of

LMX1B led to significant reduction in the percentage of TUNEL positive cells (Fig. 6C and 6D). It is also notable that overexpression of LMX1B per se did not lead to increased viability of SH-SY5Y neurons. Our results, therefore, show that LMX1B is capable of rescuing SH-SY5Y cells from MPP^+ mediated cell death.

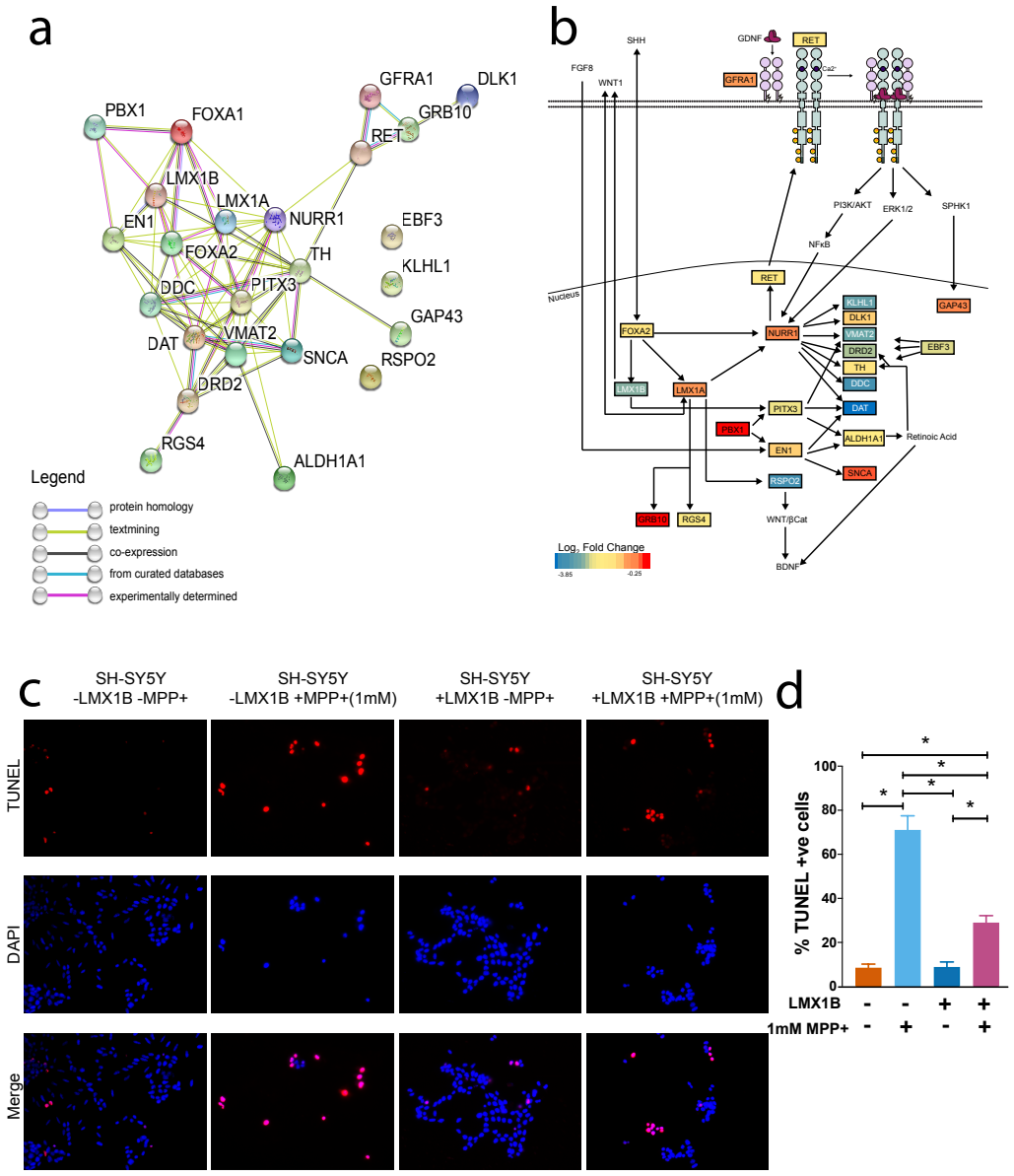

Figure 6: **LMX1B overexpression rescues SH-SHY5Y from MPP^+ toxicity.(a) String analysis of significantly perturbed midbrain dopaminergic neuron development, differentiation and maintenance genes. (b) Summary of role of genes involved in development and maintenance of SNpc dopaminergic neurons that were differentially expressed in human PD as derived from literature. (c) Representative images of SH-SY5Y neurons depicting the presence of TUNEL positive cells (Red) and DAPI positive cells (Blue) after overexpression of LMX1B and treatment with 1mM MPP^+. (d) Quantification of percentage of TUNEL positive cells as normalized to the total number of DAPI positive cells. n=28-30 images, 8-10 images taken per coverslip taken over three independent coverslips. Data represented as mean $\pm$ SEM. One-way ANOVA with Tukey's test for multiple comparison was performed to compare the different groups. represents $p<0.05$.**

Discussion

In this study, global changes in the transcriptome were studied in precisely dissected substantia nigra pars compacta (SNpc) from MPTP mouse model of PD at early and late time points after treatment with MPTP using RNA sequencing. Further, RNA sequencing was also performed on SNpc from PD patients and age-matched controls. A single dose of MPTP resulted in a larger number of differentially expressed genes in mouse SNpc as compared to 14 days treatment with MPTP. Gene expression changes in SNpc from mice after single exposure to MPTP resulted in dysregulation of pathways related to immune and inflammatory system. However, after 14 daily doses of MPTP and in human PD, these pathways were not enriched. While Riley and colleagues have indeed reported dysregulation of several inflammatory pathways[35] , it is essential to note that the RNA-seq in their study was performed on substantia nigra which would include

contribution from the GABA-ergic neurons of pars reticulata. The present study, on the other hand, evaluates gene expression changes in specifically substantia nigra pars compacta, which could explain the observed difference.

When we compared the transcriptomic changes observed in 14 days MPTP mouse SNpc with human PD, we found similarity in terms of downregulation of the common genes involved in dopaminergic phenotype maintenance. While few dysregulated pathways are common between MPTP model and human PD, the differences are predominant reinforcing the limited overlap of the molecular pathways involved in neurodegeneration.

Two striking observations of this study are the lack of enrichment of genes involved in inflammatory and immune response and the downregulation of an entire ensemble of genes in SNpc from PD subjects that are well characterized for their role in maintenance of dopaminergic phenotype, viz. FOXA1, RSPO2, KLHL1, LMX1B, EBF3, PITX3, RGS4, ALDH1A1, RET, FOXA2, EN1, DLK1, GFRA1, LMX1A, NURR1, SNCA, PBX1 and GRB10 in human PD[36–43] . The downregulation of these genes could presumably affect the expression of genes that define the dopaminergic phenotype, such as DAT, TH, DRD2 and DDC. GAP43 has been implicated in neurogenesis and its role in survival or maintenance of dopaminergic neurons is not clear[44,45] .

A summary of the functions of genes involved in development, differentiation and maintenance of dopaminergic neurons is presented in figure 6D. Sonic hedgehog (SHH), WNT1 and FGF8 are important early genes that induce the expression of FOXA2, LMX1A, LMX1B and EN1 during the earliest stages of midbrain dopaminergic neuron development, which further drive the expression of NURR1 and PITX3 and thereby lead to expression of TH, VMAT2, DRD2, DDC, DAT and ALDH1A1 that define the

dopaminergic neuron phenotype[46–52] . Further, studies have implicated the role of genes such as RGS4, GRB10 and RSPO2 that are downstream LMX1A in midbrain dopaminergic neurogenesis and differentiation[53,54] . Similarly, there is evidence that EBF3 may lie upstream of NURR1 and PITX3[55] and PBX1 is upstream of PITX3 and EN1[56–58] and may play an important role in dopaminergic neuron development. Moreover, the expression of NURR1 and PITX3 is induced by GDNF/RET signaling and has been shown to be important for protection of dopaminergic neuron[42,59] . It is also important to note that several studies have emphasized on the strong co-ordination among the members of this pathway such as NURR1 and FOXA2, PITX3 and EN1, NURR1 and EN1 and FOXA2 and LMX1A and B[56,57,60–62] .

While these genes are indispensable for the development of midbrain dopaminergic neurons, fewer studies have assessed their function in the adult SNpc. EN genes have been reported to be essential for survival of midbrain dopaminergic neurons and regulate the expression of α-synuclein (SNCA)[63] . LMX1A/B have been shown to be important for maintenance of dopaminergic neuron phenotype through their roles in regulating normal autophagic-lysosomal pathway[64] and mitochondrial functions[65] .

In the present study, we have observed the concomitant downregulation of majority of the genes important for development and maintenance of dopaminergic neurons, which not only emphasize the role that this pathway plays in pathogenesis and progression of PD but also the interdependence of these genes in dopaminergic neuron survival. However, how these genes are downregulated so specifically in SNpc from PD subjects and their roles in the adult SNpc needs to be addressed. Importantly, we demonstrated that the overexpression of LMX1B, which is an upstream regulator of the midbrain dopaminergic neuron differentiation pathway, is able to rescue cell death as a result of

MPP^+ toxicity in the SH-SY5Y neuron cultures suggesting that a revival of this pathway could potentially rescue neurodegeneration in PD.

While greater emphasis has been given to addressing the motor dysfunctions observed in PD, management of non-motor symptoms of PD including sleep and autonomic dysfunction, and psychiatric symptoms is increasingly becoming a larger burden. Interestingly, review of genome wide association studies (GWAS) revealed the association of ALDH1A1[66] and DRD2[67] with schizophrenia. DRD2 is also associated with alcohol consumption[68] and sleep deprivation[69] . NURR1[70] and LMX1A[71] are associated with alcohol consumption while DAT[72] and PBX1[73] are associated with sleep duration. Further RET has been associated with cannabis dependence[74] and smoking behavior[75] . Thus, we see that several of the genes dysregulated in PD are associated with addictive behavior and impact a variety of behavioral dysfunctions which co-occur with PD. The impact of downregulation of the genes as we see in the present study would potentially have far-reaching effects beyond motor symptoms.

In conclusion, we demonstrate for the first time that an ensemble of 19 genes that govern and regulate the development of midbrain and emergence of dopaminergic phenotype in SNpc neurons are markedly downregulated in Parkinson's disease. These not only include genes that are downstream of LIM homeobox transcription factor, LMX1B (PITX3 and ALDH1A1) and LMX1A (GRB10, RGS4, RSPO2, NURR1, KLHL1, DLK1 and RET), but also other genes that are upstream such as, FOXA2 and RET and genes regulated by FGF8 such as EN1; indicating that these genes are potentially important for the maintenance of dopaminergic neurons. Interventions that could potentially upregulate the key genes that lie upstream in this cascade could protect dopaminergic neurons and thus alter the progression of the disease. Thus, identification

of this ensemble offers a new window for discovery of disease modifying therapies for PD.

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Competing Interests The authors declare that they have no competing financial interests.

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