Global DNA methylation profiling of manganese exposed human neuroblastoma SH-SY5Y cells reveals epigenetic alterations in Parkinson's disease associated genes

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Abstract

Manganese (Mn) is an essential trace element required for optimal functioning of cellular biochemical pathways in the central nervous system. Elevated exposure to Mn through environmental and occupational exposure can cause neurotoxic effects resulting inmanganism,a condition with clinical symptoms identical to idiopathic Parkinson's disease. Epigeneticsis now recognized as a biological mechanism involved in the etiology of various diseases. Here, we investigated the role of DNA methylation alterations induced by chronic Mn (100μM) exposure in human neuroblastoma (SH-SY5Y) cells in relevance to Parkinson's disease. A combined analysis of DNA methylation and gene expression data for Parkinson's disease associated genes was carried out. Whole genome bisulfite conversion and sequencing (WGBS) indicates epigenetic perturbation of key genes involved in biological processes associated with neuronal cell health. Integration of DNA methylation data with gene expression reveals epigeneticalterationsto*PINK1*, *PARK2*and *TH*, genes that play critical roles in the onset of Parkinsonism. The present study suggests that Mn-induced alteration of DNA methylation of *PINK1-PARK2* may influence mitochondrial function and promote Parkinsonism.Our findings provide a basis to further explore and validate the epigenetic basis of Mn induced neurotoxicity.

Keywords: DNA methylation, Manganese, Epigenetics, Parkinson's disease, WGBS

Abbreviations:

APC	-	Adenomatous polyposis coli
ATP2B2	-	ATPase Plasma Membrane Ca2+ Transporting 2
CXXC1	-	DNA-Binding Protein With PHD Finger And CXXC Domain
DLK1	-	Delta-Like 1 Homolog (Drosophila
DRD2	-	Dopamine receptor D2
GBE1	-	1,4-alpha-glucan-branching enzyme
Mn	-	Manganese
NRXN3	-	Neurexin 3
NSF	-	N-Ethylmaleimide Sensitive Factor
PAN2	-	Poly(A) Specific Ribonuclease Subunit
PARK2	-	Parkin RBR E3 Ubiquitin Protein Ligase
PINK1	-	PTEN-induced putative kinase 1
PPI	-	Protein Protein Interaction
SLC25A4	-	Solute Carrier Family 25 Member 4
SNCA	-	Synuclein Alpha
STUB1	-	STIP1 Homology And U-Box Containing Protein 1
TH	-	Tyrosine Hydroxylase
VDAC	-	Voltage-dependent anion-selective channel protein 1
WGBS	-	Whole genome bisulfite conversion and sequencing
YWHAZ	-	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

1. Introduction

Manganese (Mn) is naturally present in the environment and is a vital trace element required for proper functioning of cellular biochemical pathways. Although Mnis essential for human physiology, elevated exposure can elicit neurotoxic effects on the developingcentral nervous system (CNS) (Erikson et al. 2007; Tamm et al. 2008). Miners and workers of ferroalloy, smelting and metallurgy industries are occupational populations exposed to manganese (Finkelstein MM 2007; Lucas et al. 2015). Manganese containing pesticide formulations such asmaneb and paraquat are used in agriculture to treat various plant pathologies (Roede et al. 2011). Methylcyclopentadienyl manganese tricarbonyl (MMT) used as an petrol anti-knock additive also releases Mn as a combustion product in the atmosphere (Crump 2000). Recently high exposure to Mn has been reported to occur with use of psychoactive stimulantsin recreational drugs(de Bie et al. 2007).The concentration of Mn in drinking water also was found to exceed the WHO regulatory standard (400µg/L) (WHO 2006). Significant levels of Mnhave been detected in children hair samples and breast milk inpopulations residing in the vicinity of industrial steel plant (Sharma R. 2005). Therefore, the main paths of environmental exposure toMnin the general population occursthrough intake of contaminated drinking water, dietary foods and inhalation of ambient air with elevated level of manganese (Roede et al.2011; ATSDR 2012).

High occupational and environmental exposure to Mn has been reported to cause manganism,a condition with neurological symptoms similar to those of idiopathic Parkinson's disease (Settivari et al. 2013). Clinically, the symptom includes tremors, bradykinesia, rigidity,postural instability and facial muscle spasms. The low chronic level of exposure to Mn for prolonged period have been found to be associated with elevated risk of developing Parkinson's disease (Gorell et al. 2004).

Parkinson's disease is characterized by the progressive loss of dopaminergic neurons in substantianigra pars compacta (SNpc) with subsequent depletion of dopamine, impairing the execution of coordinated movements (Anumantha et al. 2012). Recent evidence suggest thatMn deposition occurs in substantianigra region and hence may be postulated to be associated with the etiology of Parkinson's disease (Park et al. 2007).

Epigenetics entails the study of heritable changes in gene expression that occur independent of modifications to nucleotide sequences (Schnekenburger M 2007). Control of gene expression is essential for normal physiological functioning of cell and the epigenetic machinery has an important role in regulating these processes. Epigenetic regulation includes of DNA methylation, histone modifications and miRNA expression. The involvement of epigenetic deregulation in the cause of various neurodegenerative disorders such as Parkinson's, Huntington Alzheimer and other mood disorders has been suggested (Migliore L 2009). Environmental toxicants that induce epigenetic alterations have been implicated in the susceptibility and progression of environmentally mediated chronic diseases (Baccarelli and V. Bollati 2009; Anumantha et al. 2012).

The generation of oxidative stress through mitochondrial accumulation of Mn and subsequent mitochondrial respiratory dysfunction is thought to be primarily responsible for neurotoxic effects of manganese (McCubrey JA and Lahair MM 2006). For example, Mn has been reported to cause apoptotic cell death via oxidative stress in the human neuroblastoma SH-SY5Y cell line(Stephenson et al. 2013). Exposure to heavy metals such as As, Pb, Cd and Ni

generates reactive oxygen species that may alter the level of DNA methylation in rat and other animal models(Martinez R 2011).

DNA methylation alterations occur primarily at CpG sites through addition of a methyl group to the 5' position of cytosines. Around 70% of the CpGs sites are constitutively methylated in human genome, while unmethylatedCpGs are generally located in CpG islands associated with promoter regions of genes (Martin C 2007). Methylation modifications at CpG sites can induce conformational changes to chromatin and thereby modulate accessibility of gene promoter regions to the transcription machinery (Orphanides G 2002).

Human population is inevitably exposed to manganese in the environmental and occupational settings. It is evident from the epidemiological studies and available literature that populations exposed to low chronic levels of Mn have a propensity to develop idiopathic Parkinson's disease (Kim et al. 2002). However, the hypothesis involving the potential role of epigenetic mechanisms in manganese induced neurotoxicity has not been explored (Tarale et al. 2016).

In the present study we investigated the impact of chronic Mn exposure on whole genome DNA methylation levels and profiling of differentially methylated genes. We used the dopaminergic human neuroblastoma (SH-SY5Y) cell line as a model to explore the role of DNA methylation alterations induced by Mn and its possible association withParkinson's disease. In support of our DNA methylation data, we also correlated differentially methylated regions with expression levels ofParkinson's disease associated genes. The results of the present investigation provide insight how epigenetic mechanisms may promote manganese-induced idiopathic Parkinson's disease.

2. Material and Methods

2.1 Cell line

Human neuroblastoma (SH-SY5Y) cell line was procured from National Center for Cell Science (NCCS), Pune, India. Cells were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 50 µg/mL streptomycin, and 25 µg/mL Fungizone. Cells were maintained at 37°C in a humidified atmosphere with 5% CO2.

2.2 Chronic Mn^{2+} exposure

SH-SY5Y cells were seeded in $25mm^2$ flask (5×10^5 cells/ml) and grown up to 70% confluence level. After achieving the 70% confluence level, cells were exposed to 100 µM of Mn^{2+} in the form of $MnCl_2$ (Sigma Aldrich, USA). The dose of $Mn^{2+}$for exposure in this study was selected based on the published research articles and preliminary studies (Bornhorst et al. 2013; Stephenson et al. 2013). Chronic exposure of $Mn^{2+}$was given for the period 30 days, three controls and three exposed flask were maintained during the period of exposure. Medium was subsequently changed at every third day and after each passage throughout the exposure period. Cells were treated with Mn^{2+}after each passage as the cells get adhere to the flask.

2.3 Whole-genome (DNA) methylation sequencing and data processing

At the end of exposure period, cells were harvested for the whole genome DNA methylation sequencing and analysis as follows.

2.3.1 Isolation of genomic DNA

SH-SY5Y cells were harvested and suspended in lysis buffer (500µl of TNE buffer, 25 µl of 20% sodium dodecyl sulfate (SDS), 7.5 µl of proteinase K (20mg/ml)). Samples were then incubated at 37°C for 3 hrs. After 3 h incubation, 250µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to cell lysate and mixed well, further samples were kept for 30 min at room temperature. Later samples were centrifuged at 14000g for 15 min. After centrifugation aqueous layer was collected to new eppendorf tubes and DNA was precipitated with 90% ethanol and 25µl of 0.3 M sodium acetate. Samples were centrifuged at 14000g for 10 min to pellet out the precipitated DNA. Precipitated DNA was washed with 70% ethanol and again re-centrifuged. Finally DNA was air dried and re-suspended in DNase free water. Quantification of genomic DNA was carried out using ND8000Nanodropspectrophotometer.

2.3.2 Bisulfite conversion

EZ DNA Methylation GoldTM Kit (Zymo Research) was used for the bisulfite conversion of 20µl (200ng/µl) of genomic DNA. 20µl of each DNA sample was mixed with 130µl of CT conversion reagent and programmed at thermal cycler as; 98º C for 10 min, 64º C for 2.5 hrs, 4º C for 20 hrs. After the incubation, 600µl M binding buffer was added to tubes and transferred to Zymo spin IC column. Columns were centrifuged at 10000g for 30 sec. Following centrifugation, columns were washed with M wash buffer. Later 200µl of M desulphonation buffer was added to columns and incubated at room temperature for 20 min followed by centrifugation at max speed. Columns were then washed with M buffer and bisulfite converted genomic DNA was eluted with M elution buffer.

2.3.3 Whole genome bisulfite sequencing

Whole genome bilsulfite sequencing was carried out using EpiGnome^{TM} Methyl-Seq Kit on IlluminaHiseq 2500. 100ng of bisulfite converted genomic DNA samples were mixed with 2µl DNA primer and incubated for 5 min at 90º C. After the incubation, 4µl of EpiGnome DNA synthesis premix, 0.5µl of 100mM DTT and 0.5µl of EpiGnome polymerase was added to samples was and programmed on thermal cycler (25°C for 5 min, 42ºC for 30 min, 37°C for 2 min). Later 1.0µl of exonuclease was added to each samples and reaction was programmed on thermal cycler (37°C for 10 min, 95°C for 3 min, 25°C for 2min). At the end of programme, 8µl of Terminal Tagging master mix (7.5µl of EpiGnome^{TM} Terminal Tagging premix and 0.5µl DNA polymerase) was added to each sample tubes and programmed on thermal cycler (25°C for 30 min, 95°C for 3 min and hold at 4°C). Prior proceeding DNA library preparation, DNA was purified using AMPure XP system to yield di-tagged DNA. EpiGnome Index PCR Primer kit was used to generate whole genome bisulfite library. To 22.5µl of di-tagged DNA, 24µl of Failsafe PCR premix E, 1.0µl of EpiGnome forward primer, 1.0µl of EpiGnome reverse primer and 0.5µl of Failsafe PCR Enzyme was added and programmed on thermal cycler for 10 cycles as 95°C for 30 seconds, 55°C for 3 min, 68°C

and final extension at 68°C for 7 mins. Whole genome bisulfite library was then purified by using AMPure XP system.Totalyield of WGB library was quantified using Qubitfluorometer and size distribution of WGB library was analyzed using2100 bioanalyzer (Agilent). Whole genome bisulfite sequencing (Paired End) of bisulfite library was carried out on IlluminaHiseq 2500 using Illumina SBS and Cluster kit.

2.3.4 Bismark alignment

Whole genome bisulfite sequencing (WGBS) was carried out to generate the DNA methylation map of SH-SY5Y cell line chronically exposed to Mn (100µM). Two biological replicates, each of control and exposed SH-SY5Y cell line were sequenced. Bismark alignment in control and exposed samples mapped an average of 62% of reads the genome. The 90% of the sequenced data passed Q30 phred quality score. According to Bismark alignment mapping efficiency within a range of 60-68% was considered as the best alignment percentage for reads of>80 base pairs of length. The whole genome bisulfite sequencing (WGBS) data was submitted to National center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository and can be accessed (SRP075314; SRP075316; SRP075243; SRP075313). The publically submitted whole genome DNA methylation data may be further explored using other bioinformatics tools to obtain additional information in relevant to manganese neurotoxicity

2.3.5 Methylation analysis and identification of differentially methylated genes

The base calling pipeline (HiSeq Control Software 2.2.38) was used for demultiplexing prior to the generation of fast-q-sequence files, i.e by separating the libraries according to their indexes. Passed filter (PF) is a cluster that fulfills the default Illumina quality criteria; % of bases (PF) with a quality score greater or equal to 30 was used as a cutoff to remove sequences produced from clusters with low signal to noise ratio (e.g. overlapping DNA clusters). A 6-8 bp pair DNA sequence tag found in adaptor sequence to uniquely identify each library; used at demultiplexing step. This filtered reads are aligned to the Human Genome reference (hg19/GRCH37 Assembly). Bismark tool (Version – V 0.10.1) was used for the alignment of bisulfite converted genome sequences. Each cytosine (C) present in raw reads was extracted from alignment output file (SAM file) using Bismark. The position of every cytosine (C), depending on cytosine (C) context (CpG, CHC and CHH) methylated cytosine (C) was labeled as "+" (forward read) while unmethylated cytosine (C) labeled as "-" (reversed read). Every methylated cytosine (C) was annotated against human reference genome using Varimat tool. aEach cytosine (C) was tracked to its original location in the genome based on its chromosome position to identify differentially methylated regions. Methylation difference between control and Mn^{2+} exposed cells was calculated by using DMAP (Differential Methylation Analysis Package for RRBS and WGBS data). We screened for differentially methylated genes between Control and Mn-treated samples having methylation difference above 0.1 (hyper orhypomethylation). The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (https://david.ncifcrf.gov/) was used for functional annotation clustering of differentially methylated genes.

2.4 Gene expression profiling for Parkinson's disease associated genes

SH-SY5Y cells were harvested at the end of exposure period for the isolation of total RNA. Trizol reagent (Invitrogen) was used for the isolation of total RNA. Quantification of total RNA was carried out using NanoDrop 8000 spectrophotometer. 2000ng of RNA was converted to cDNA using the RT^2 first strand kit (Qiagen, Germany) as per the manufacturer instructions. The Human Parkinson's Disease RT² Profiler PCR Array (Qiagen, Germany) profiles the expression of 84 key genes directly or potentially involved in Parkinson's disease . Each RT² Profiler PCR array contains 12 controls that were used for the normalization of RT-PCR results. Applied Biosystems7300 real time PCR was used for the relative quantification and RT-PCR was performed as per the manufacturer protocol. ΔΔCt method of relative quantification was used for the calculation of fold change and interpretation of control wells.

3. Results

3.1 Methylation profiling

We scanned the genome of Mn-treated and untreated SH-SY5Y cells for cytosine methylation by mapping methylated and unmethylated cytosines to a virtual, bisulfite converted human genome. Chronic exposure to manganese resulted in overall slight increase in the percentage of methylation density at CpG sites from 70.4%to 73.6%(Figure 1A and B). Methylation of cytosines has also been detected at non-CpG sites in neuronal DNA (Ryan Lister, Eran A. Mukamel, Joseph R. Nery, Mark Urich et al. 2013). We therefore, examined our WGBS data setsfor the presence ofmethylation events at CHG and CHH sites(H = A/C/T). In context of non-CpG sites anincrease in methylation levels was detectedat CHG sites,from 3.4% to 19.7%(Figure 1C). Similarly,at CHH sites, methylation was markedly elevated, from 3.7%to 25.4%(Figure 1D).

The expression of genes is influenced by cytosine modifications. To map transcriptional start sites (TSS) and to identify genes associated with TSS regionson their original locations in chromosomes, we used VariMAT to annotate the observed cytosine methylation events. This gives the list of differentially methylated genes that are linked with altered state of CpG methylation.

3.2 Differentially methylated gene analysis

We examined if a genome-wide trend for methylation of TSS could be detected. Methylation densities of CpG sites within 5 kilobase (kb) regions upstream and downstream of TSS were determined with 200 base pair (bp) genomic bins using our WGBS data. This analysis suggested a subtle trend towards methylated TSS and increased levels of cytosine modification downstream, in the gene bodies of the Mn exposed samples (Supplementary figure 2).The WGBS data were further analyzed for identification of genomic sequences associated with a distinct CpG methylation status. Chromosome wise analysis identified 24868 differentially methylated genes. Genes from this list were further shortlisted based on the p_value (<0.05) to obtain a list of around 10213 differentially methylated genes at statistically significant level (Supplementary file 1). The list of genes was again screened, based on <0.1% methylation difference/change, yielding 6846 differentially methylated regions of genes. The heat map showing the differential methylation status for genes with >0.1methylation difference is given in supplementary figure S1.The

Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 was used for functional annotation clustering of differentially methylated genes. The hypermethylated and hypomethylated genes were separately analyzed using theDAVID database for functional annotation and clustering. DAVID based analysis shows that the hypermethylated genes are involved in metal ion binding (25%), cytoskeleton (16%), chromatin modification (12%), regulation of transcription (7%), apoptosis and iron binding (13%) (Figure 2A). The hypomethylated genes are involved in regulation of signal transduction (38%), transcription (15%), neuron differentiation and development (5%), synaptic transmission and MAPK signaling pathways (9%) (Figure 2B).The important hypermethylated genes regulating the synaptic transmission are PINK1, PARK2, SMAD7, VGF, DBH, GDNF, TH, GRIK3, and NGF. The genes responsible for regulating the apoptosis such as MGMT, CASP3, CARD14, MAPK8, MAP3K7, NGFR, YWHAZ were also found to be hypermethylated. The several inflammatory genes, including HRH1, IGF2, ITGB6, MAP2K3, YWHAZ, LYN and MASP1were found to be hypomethylated. In summary, DAVID analysis reveals that manganese exposure alters the DNA methylation status of genes involved in the important biological pathways. The perturbation in the regulation of biological pathways via methylation alteration of associated genes may be responsible for the neurotoxic mechanism of manganese.

3.3 Correlation of differentially methylated genes with knowngenes inParkinson 's diseaseRT² Profiler array

The DNA methylation profiling approach identifies genes potentially involved in pathways related to Parkinson's disease. In order to correlate and validate the differentially methylated genes with their relative level of gene expression, we used the Human Parkinson's Disease RT² Profiler PCR Array (Qiagen, Germany). From a total of 84 genes screened with this PCR array, we found that34 genes had significantly diminished expression levels ($p<0.05$) in the Mn treated SH-SY5Y cells. To assess if this set of down-regulated genes is enriched for certain functional pathways or networks, the DAVID database was used for annotation and clustering based on the gene ontology. The identified 34 genes were found to be involved in three biological pathways, including neuronal development and differentiation (17 genes), dopamine metabolic process (8 genes), and ubiquitin mediated proteolysis (5 genes) (Figure 3).Important genes associated with these pathways are ATP2B2, DRD2, NRXN3, SLC25A4, SNCA, TH, VDAC, PARK2, PINK1, NSF, PAN2, STUB1. These three pathways are known to play a role in the pathology of Parkinson's disease. The same pathways that we identified to be deregulated using the RT² expression profiler were also flaggedby a DAVID analysis of our list of differentially methylated genes.The differentially methylated genes identified in the WGBS screen were compared to the list of 34 genes identified in the RT^2 PCR expression profiler array for human Parkinson's disease and additionally with genes that have been reported in the literature to be associated with Parkinson's disease. This type of comparison could indicate a probable connection between manganese induced DNA methylation alteration and changes in expression of Parkinson's disease associated genes. Based on this analysis we identified 119differentially methylated genes from our WGBS data set,which is shown in a heat map(Figure 4).

Correlation of DNA methylation and gene expression in the present study identifies 10 genes in relevance to Parkinson's disease (APC, ATP2B2, CXXC1, DLK1, GBE1, NSF, PARK2, PINK1, TH, and YWHAZ) (Figure 5). Among these 10 genes; TH, PARK2 and PINK1 are particularly recognized as important players in the etiology of Parkinson's disease as they have a significant role in the proteosomal degradation, protein kinase activity and dopamine biosynthesis (Satake et al. 2009)Table 1 gives the list of 10 genes with their status in DNA methylation profile and RT^2 PCR gene expression analysis. The methylation alteration of these critical genes shows the probable association between manganese exposure and Parkinson's disease at an epigenetic level. The STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) is mainly devoted to documenting known and predicted functional protein-protein interactions. Our STRING analysis reveals a significant biological association between the genes as group with PPI (p-value<0.0002) (Figure 6). The major biological process (GO) altered in connection to Parkinson's disease has been highlighted in the figure 6.

4. Discussion

Epigenetics has gained recognition as a biological mechanism that is associated with the manifestation of various human diseases. The interaction between gene and environment is thought to be, in part at least, mediated via epigenetic mechanisms. Epigenetic changes have already been implicated in certain cases of Parkinson's disease (Moyano et al. 2011). There is growing evidence suggesting a link between manganese exposure and Parkinson's disease (Racette et al. 2012). However there are limited studies that aimed to determine the connection between manganese exposure with deregulation in epigenetic mechanisms leading toParkinson's disease (Nannan et al. 2016).

The human neuroblastoma cell line (SH-SY5Y) has been used as *ex vivo* cell model to investigate the neurotoxic mechanism of manganese (Li et al. 2010). In the present study we chronically exposeda neuroblastoma cell line to Mn^{2+} (100µM) for a period of 30 days, followed by WGBS and gene expression analysis for Parkinson's disease associated genes. Hypermethylation of CpG sites within gene promoter regions generally correlates with repression of gene expression(Nan et al., 1998). In contrast, non-CpG cytosine modificationshavebeen detected in active genomic regions in human and mouse brain (Lister et al. 2013; Guo et al. 2014). In the present study we observed an increase of non-CpG methylation, particularly at CHH sites (figure 1C and 1D). The methyl CpG binding protein 2 (MECP2) plays an essential role in normal neuronal development (Chahrour et al. 2009) and appears to modulatetranscription by interaction with both methylated CpG sites and methylated mCAsites (Matthew et al. 2013). The overall methylation increase at both CpG and non-CpG sites observed in the present study may be associated with analtereddistribution of MECP2.

The WGBS data was further analyzed to identify specific genes with altered methylation. The list of significantly hypermethylated and hypomethylated genes was submitted to Database for Annotation, Visualization and Integrated Discovery (DAVID) for functional annotation and clustering (Dennis et al. 2003). DAVID analysis indicates the involvement of these differentially methylated genes in multiple important biological pathways such as regulation of

neuronal differentiation and development, synaptic transmission, signal transduction, inflammation and programmed cell death. Exposure to manganese has been reported to alter the DNA methyaltion status of various CpG loci during the neurodevelopmental process (Maccani et al. 2015). The hypomethylation of inflammatory nitric oxide synthase gene (iNOS) was reported responsible for elevated signs of Parkinsonism among the Mn miners (Susan et al. 2014). DAVID based functional classification of genes in the present work also identifies the hypomethylation of inflammatory genes.

It is important to recognize there may not be a direct correlation between the DNA methylation status and expression profile of genes (Jones 2012). Hence it becomes important to integrate DNA methylation data with gene expression profiles. In the recent study combined analysis of gene expression in addition to the DNA methylation was carried out for a select number of genes. DAVID analysis identifies neuronal development, synaptic transmission and apoptosis as common pathways involved in Mn induced Parkinsonism.

Clinically, Parkinson's disease is accompanied by motor dysfunction because of dopamine depletion occurring as a result of dopaminergic neuronal degeneration within the substantianigra pars compacta (SNpc) (Ammal Kaidery et al. 2013).Five genetic loci, including α-synuclein, parkin, PINK1, DJ-1, and LRRK2 are the most extensively studied and considered as critical risk factors for the onset of sporadic Parkinson's disease (Rochet and Hay 2012). In the present study activity of two genetic loci, parkin and PINK1 was found to be altered via hypermethylation upon manganese exposure.

Tyrosine hydroxylase (TH) has an important role in dopamine biosynthesis. Mn exposure has been found to alter the activity of TH, both in dopaminergic neuronal cells and a Zebrafish model of manganism (Zhang et al. 2011; Bakthavatsalam et al. 2014). The result of the present investigation suggests that cytosine methylation plays a role in modulating TH gene activity and may contribute to diminished expression levels, which is in agreement with previous studies on Mn neurotoxicity (Bakthavatsalam et al. 2014).. However, our results require further validation and testingin other animal models.

Parkin/PARK2 is an autosomal recessive gene and mutations in one of the alleles has been shown to cause the clinical features of Parkinsonism and as risk factor for early onset of Parkinson's disease (EOPD) (Periquet M et al. 2003; Padmaja et al.2012)The present study suggests that hypermethylation of PARK2 with subsequent reduction in gene expression may be another molecular mechanism that potentiates Mn toxicity via loss of control over Divalent Metal Transporter (DMT1) mediated Mn transport(Roth and Garrick 2003; Higashi et al. 2004).

PINK1 is a serine/threonine kinase with neuroprotective function that localizes to mitochondria (Petit et al. 2005; Silvestri et al. 2005). We found PINK1 expression to be decreased in the present study, presumably through hypermethylation of the gene. Perturbation in PINK1 function through mutation has been reported to cause impairment of mitochondrial function, synaptic transmission and elevated sensitivity to oxidative stress (Kitada et al. 2008). PINK1 has also been found to interact with polycomb histone-methylation modulator (EED/WAIT1) and regulate gene expression during SH-SY5Y differentiation (Berthier et al. 2013).

PARK2 and PINK1 have been identified as key genes responsible for early onset of Parkinsonism via loss of mitochondrial quality control (Noriyuki Matsuda 2015). Mitochondrial quality control is an essential function of healthy neurons ensuring cell survival, while dysfunction of complex I has been reported to induce Parkinsonism and sporadic Parkinson's disease (Schapira 2008). Parkin/PARK2 is selectively targeted to depolarized mitochondria to facilitate the removal of damaged mitochondria through ubiquitination of outer mitochondrial substrate (Narendra et al. 2010). PINK1 kinase activity is responsible for both activation and recruitment of PARK2 to mitochondrial membrane (Youle 2014). Initially, PINK1 phosphorylate ubiquitin on depolarized mitochondria that function as an activator of PARK2 activity through repression of auto-inhibiting activity of PARK2 (Trempe et al. 2013). The subsequent events of ubiquitin-phosphorylation act as a signal for PINK1-PARK2 mediated mitophagy based removal of depolarized mitochondria (De-Chen et al. 2015). The findings suggest that PINK1-PARK2 together have a vital role in regulating mitochondrial quality control and thereby appear to prevent the onset of Parkinsonism (Narendra, et at. 2012; Youle 2014). DNA methylation studies in manganese exposed mouse model recently reported alteration to several mitochondrial genes (Yang et al. 2016).Epigenetic down-regulation of both PINK1-PARK2 through hypermetylation in the present study supports the previous findings and supports the concept that mitochondrial dysfunction might be responsible for Mn induced Parkinsonism.

This is one of the first studies to reveal manganese induced alterations in DNA methylation. The results indicate the influence of chronic manganese exposure on epigenetic de-regulation of Parkinson's disease associated pathways. Our data, along with those communicated by Yang and colleagues (Yang et al. 2016) signify the importance of considering the role of epigenetic mechanisms in Mn induced neurotoxic effects. These results require validation and replication in other animal models of Parkinson's disease.

5. Conclusion

The study shows that chronic manganese exposure on dopaminergic human neuroblastoma cells (SH-SY5Y) induces global alteration to DNA methylation levels of genes involved in biological pathways, including neuronal development and differentiation, synaptic transmission, signal transduction, and inflammation. Mitochondrial dysfunction is known to be involved in Parkinsonism and Mn has also been shown to alter mitochondrial function. The present study suggests an epigenetic alteration of PINK1-PARK2 which might contribute to the Mn mediated mitochondrial dysfunction and probably in the etiology of Parkinson's disease. A better understanding of Mn induced neurotoxicity and an altered epigenetic landscape that comes along with Mn exposure could lead to the identification of new prognostic and therapeutic epigenetic targets.

Figure legends

Figure 1. Overall methylation densities (%) from the single-base resolution methylomes (WGBSdata) were determined for(A)cytosines atCpGsitesand (B)cytosines atnon-CpG sites.Non-CpG methylationof cytosines is categorized in the context of CHG and CHH sequences, where H = A/C/T. The frequencies of these cytosine methylation events for different CHG and CHH sequence combinations is shown for the control(C) and the manganese-treated sample (D). The WGBS-approachgenerates sequence data for both, the reference sequenceof the genome ('Watson' DNA strand) and the sequence of the complementary DNA strand ('Crick'DNA strand). Methylation densities do not show any obvious strand bias.

Figure 2. DAVID database was used for the Gene Ontology (GO) functional annotation cluster analysis. The figure depicts the major biological pathways associated with (A) Hypermethylated and (B) Hypomethylated genes.

Figure 3. Gene Ontology (GO) functional annotation cluster analysis was performed using DAVID database for differentially expressed genes identified in PCR array for Human Parkinson's Disease.

Figure 4. Heatmap clustering of 119 genes screened based on involvement in Parkinson's disease pathways. Red and green colors represent the high and low level of DNA methylation.

Figure 5. Relative gene expression level of the selected genes in Human Parkinson's Disease RT² Profiler PCR Array.Error bar represent the standard error (SE) and * indicates statistical significance ($p<0.05$).

Figure 6. The STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) based analysis shows the functional association between the genes. Protein-protein interaction (PPI) enrichment p-value, highlighted in blue box indicates significant interaction and biological association between genes as a group. Biological process (GO) and genes in network highlighted in red box represent the process deregulated in Parkinson's disease pathway.

Table 1. Table depicting the genes identified on correlation between genes in DNA methylation and RT2 profiler array for Parkinson's disease. Table shows the biological function of the genes listed

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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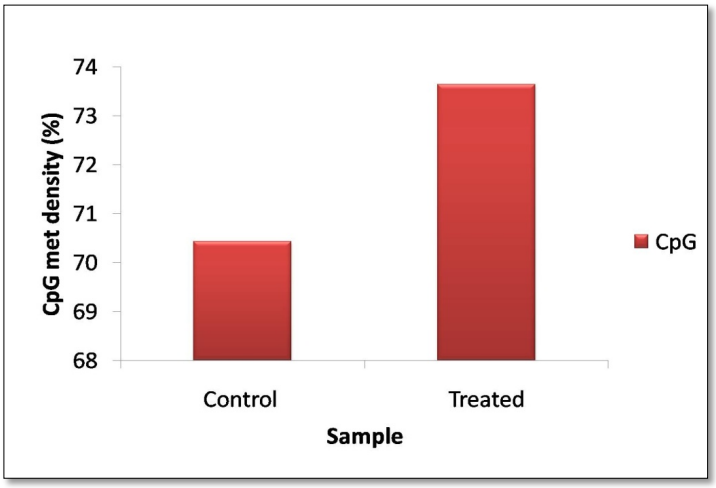
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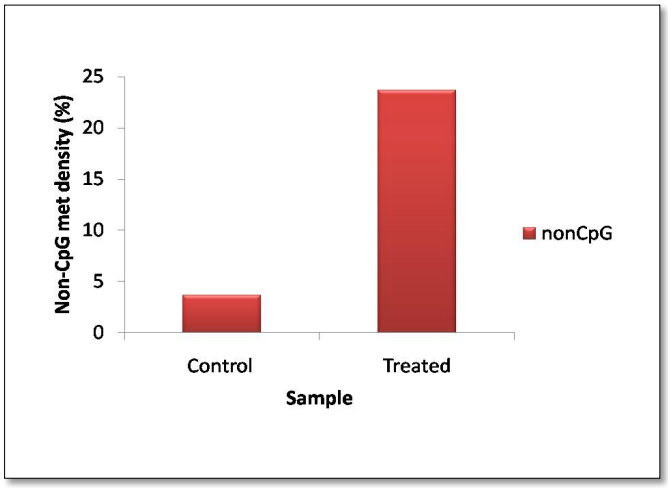
CpG
Control
Treated
Sample

(B)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nonCpG
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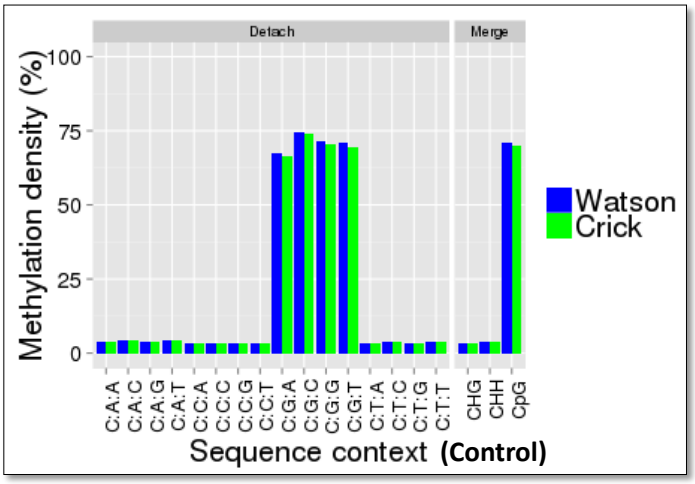
(C)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Methylation density (%)
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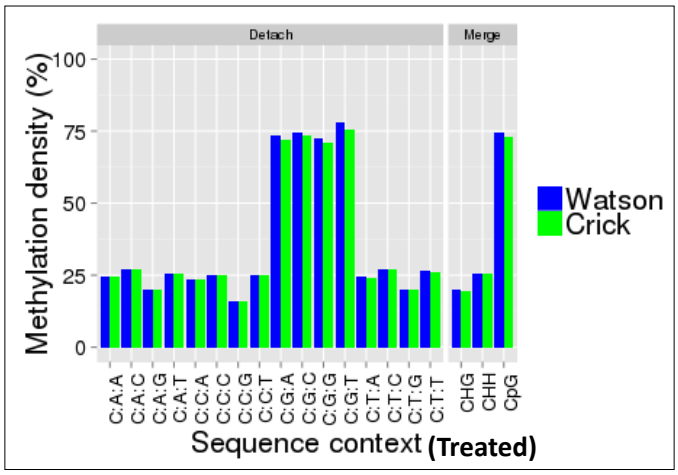
CHG
CHH
CpG
Sequence context (Control)

(D)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Methylation density (%)
Watson
Crick
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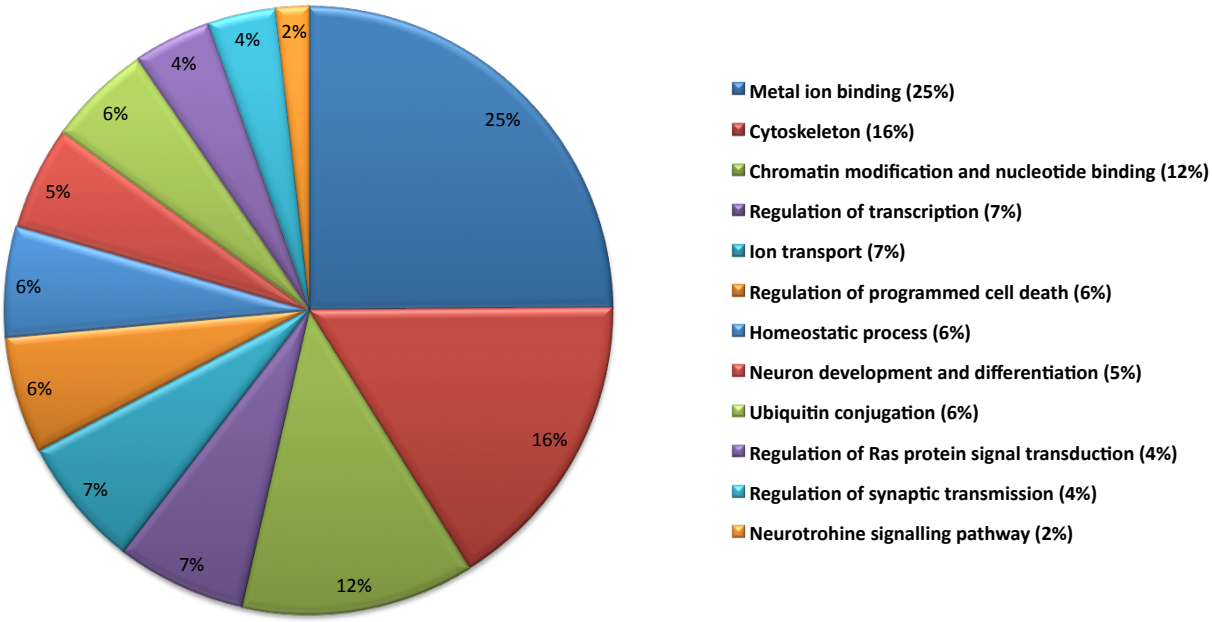
CHG
CHH
CpG
Sequence context (Treated)

(A)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Metal ion binding (25%)
Cytoskeleton (16%)
Chromatin modification and nucleotide binding (12%)
Regulation of transcription (7%)
Ion transport (7%)
Regulation of programmed cell death (6%)
Homeostatic process (6%)
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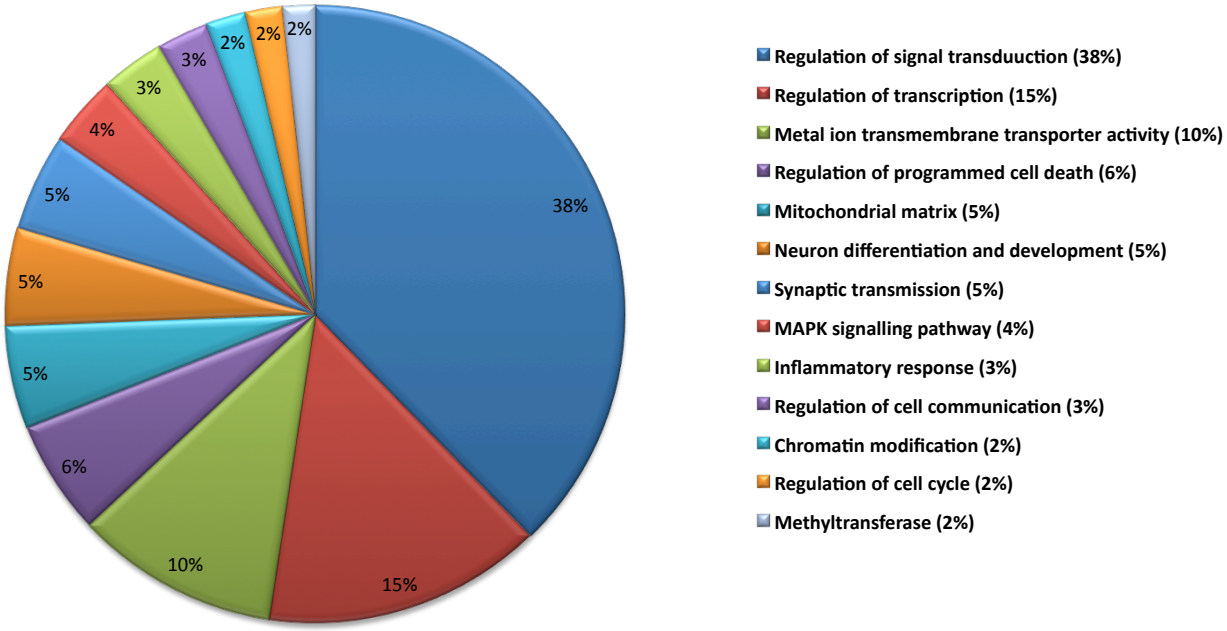
(B)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Regulation of signal transduuction (38%)
Regulation of transcription (15%)
Metal ion transmembrane transporter activity (10%)
Regulation of programmed cell death (6%)
Mitochondrial matrix (5%)
Neuron differentiation and development (5%)
Synaptic transmission (5%)
MAPK signalling pathway (4%)
Inflammatory response (3%)
Regulation of cell communication (3%)
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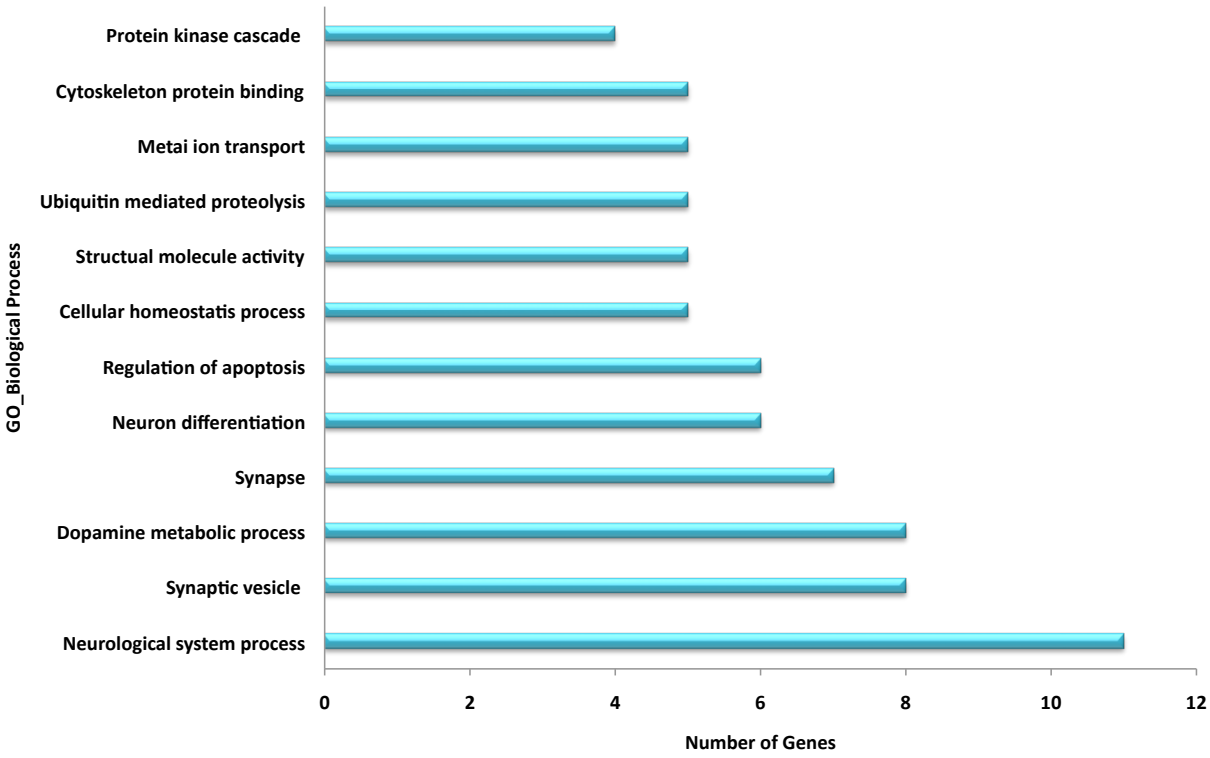
Regulation of cell cycle (2%)
Methyltransferase (2%)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Ubiquitin mediated proteolysis
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Regulation of apoptosis
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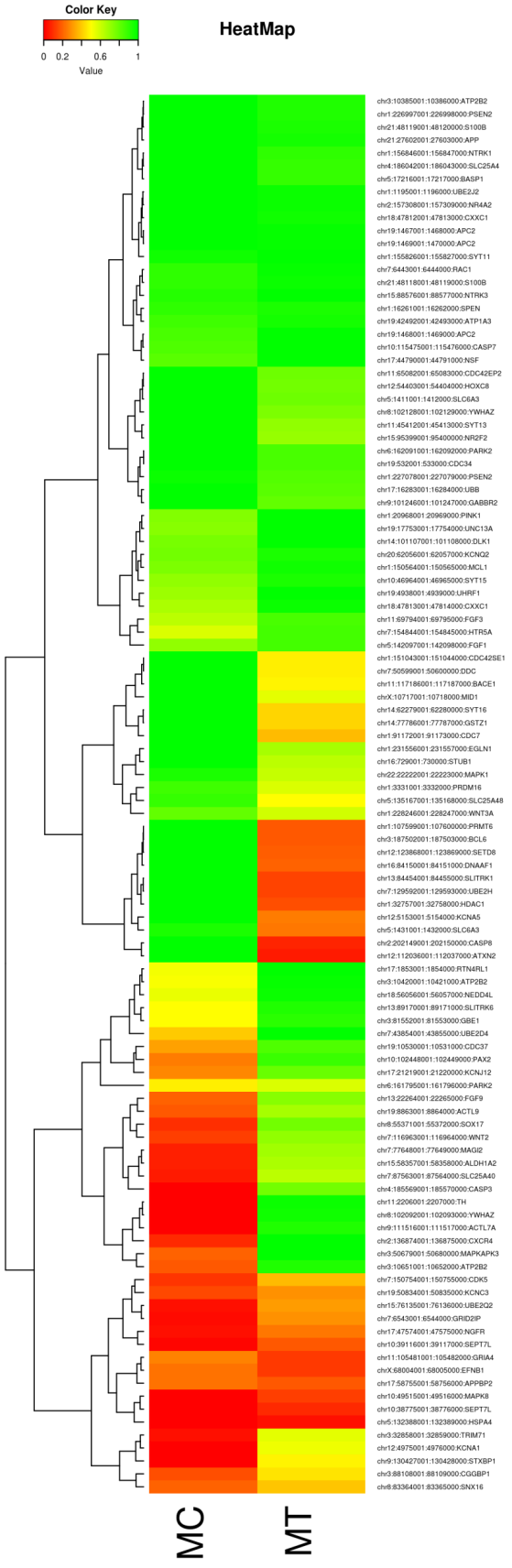
Number of Genes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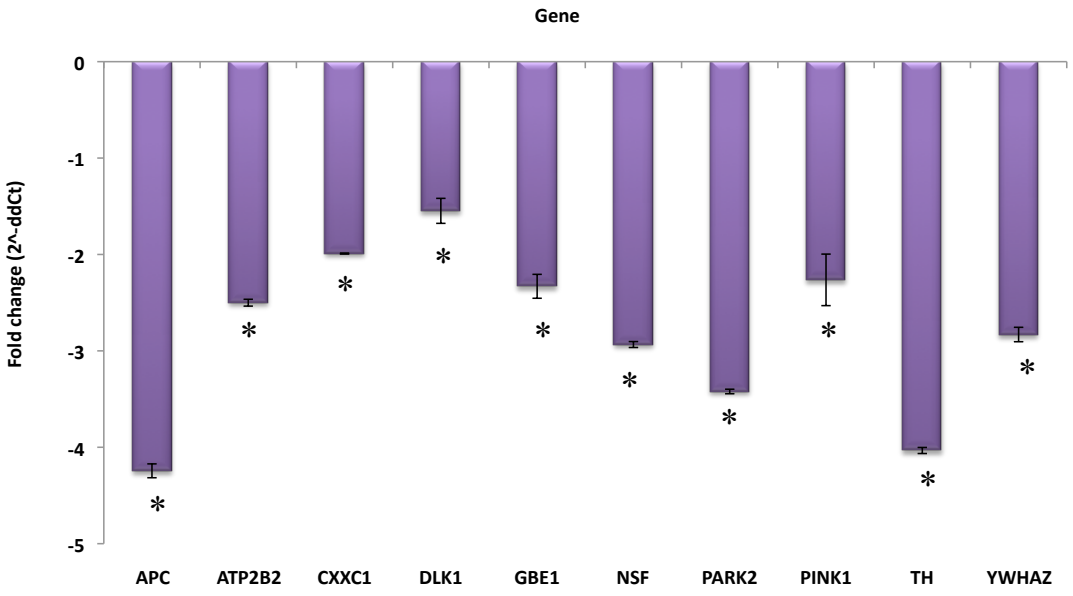
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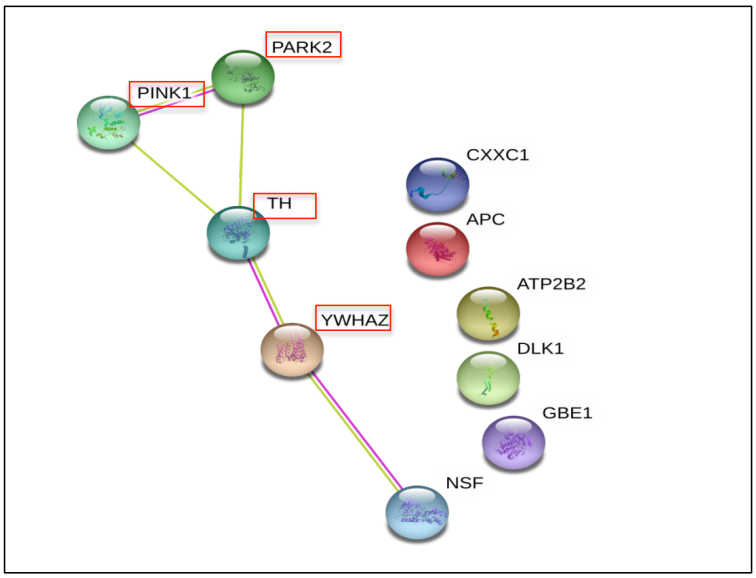
TH
YWHAZ



Network Stats

number of nodes: 10
number of edges: 5
average node degree: 1
clustering coefficient: 0.833

expected number of edges: 1
PPI enrichment p-value: 0.000273
your network has significantly more interactions than expected (what does that mean?)

Functional enrichments in your network

Biological Process (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0071287	cellular response to manganese ion	2	0.0148
GO:1903204	negative regulation of oxidative stress-induced neuron death	2	0.0148
GO:1903377	negative regulation of oxidative stress-induced neuron intrinsic apoptotic signaling pathway	2	0.0148
GO:0001963	synaptic transmission, dopaminergic	2	0.0384
GO:0010042	response to manganese ion	2	0.0384
GO:0042415	norepinephrine metabolic process	2	0.0384
GO:0090140	regulation of mitochondrial fission	2	0.0384
GO:1903747	regulation of establishment of protein localization to mitochondrion	3	0.0385
GO:0014059	regulation of dopamine secretion	2	0.0452
GO:0043409	negative regulation of MAPK cascade	3	0.0452
GO:0009636	response to toxic substance	3	0.0484
GO:0097164	ammonium ion metabolic process	3	0.0484
GO:0097237	cellular response to toxic substance	2	0.0484
GO:1902176	negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	2	0.0484
GO:1903827	regulation of cellular protein localization	4	0.0484

KEGG Pathways

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
05012	Parkinson s disease	3	0.0102