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Transcriptome Analysis of Rotenone Induced Neurotoxicity in Enriched Rat Primary Ventral Mesencephalic Neurons

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Abstract

Rotenone induced neurotoxicity is being widely investigated in relation to Parkinson's Disease (PD). However the crucial molecular mechanisms involved in rotenone induced PD still remains elusive. This report details the transcriptome changes on rotenone treatment in enriched rat primary ventral mesencephalic neurons through microarray analysis. Transcriptome analysis had yielded 705 up-regulated genes and 2415 down-regulated genes. This data was further validated by quantitative real time PCR analysis. To further refine this data, association rule mining was used and a gene interaction network among 53 genes was developed. Functional characterization of the top 25 scored genes in the network was done using panther database. Interestingly TNF was the highest scored gene among them and found to be significantly down regulated on rotenone treatment. Along with TNF other inflammation related genes like Il1b, Itpr3 and TNFR2 were also significantly down-regulated on rotenone treatment. These observations suggest that down regulation of neuronal TNF might be a critical cause leading to cellular death *via* TNFR2, Il1b mediated Pi3Kinase pathway in rotenone induced neurotoxicity. Further investigation in this neuronal TNF related pathways may give novel therapeutic approaches in treatment of PD.

Keywords: Parkinson's disease; Micro array; Tumor necrosis factor; Neurodegeneration

Introduction

In-Vitro/In-Vivo treatments with rotenone are known to induce certain features of Parkinson Disease (PD) [1]. Dopaminergic neurodegeneration in substantianigra pars compacta of Ventral Mesencephalic (VM) brain region is the hall mark feature of PD [2]. Rotenone, besides affecting mitochondrial function was also reported to be affecting a variety of cellular processes like cytoskeleton stability, inflammation, oxidative stress and apoptosis [1,3,4]. All these observations were made using directed approaches studying few of the genes involved in those specific pathways. Microarray analysis of whole transcriptome is an alternative approach for identifying key genes and pathways that might not be feasible through single-gene studies. Enriched rat primary VM neurons were well characterized [5] and studied in co-relation with rotenone induced neurotoxicity [6]. In the present study primary VM neurons were analyzed for changes in their genome expression upon rotenone treatment using microarray analysis along with Association Rule Mining (ARM) [7] for discovering the relationship among genes in a large dataset.

Methodology

Animals

Pregnant female wistar rats were procured from the National Institute of Nutrition, Hyderabad, India and maintained at University of Hyderabad animal house facility.

Ethical approval: Animal experiments were carried out according to the norms of Institutional animal ethical committee, University of Hyderabad (Proposal number LS/IAEC/AKK/10/1).

Isolation and culture of VM neurons: VM neuronal culture was done following the previously reported protocol by Bollimpelli VS *et al.*, 2015. VM neurons were seeded at 3X 106 cells in 1ml of Dulbecco's Minimum Essential Media (DMEM- F12) with 10% fetal bovine serum and 1X pen strep (Gibco, NY, USA) per well in a 6 well plate coated with 0.1mg/ml Poly-D- Lysine (PDL) (Sigma chemical co, MO, USA). Cultures were incubated at 5% CO_2 and 37$^{\circ C}$. Cultures from 2nd-DIV were supplemented with 2μM of mitotic inhibitor arabinosylcytosine (Sigma chemical co, MO, USA). Rotenone was dissolved in DMSO (Sigma Chemical Co, MO, USA) and added to the culture in a single application at specified concentration for 48 h.

Microarray

VM neurons at 7th Day *In Vitro* (DIV) were treated with 15nM rotenone, and the cells were processed at 9thDIV for microarray analysis. DMSO alone treated cells at 7th DIV were harvested at 9th DIV and were considered as control. The samples were processed for microarray analysis at Genotypic Technology Pvt Ltd, Bangalore, India, with whole rat genome microarray kit provided by Agilent. A fold change of 0.6 was used to detect the up-regulation and down-regulation with a geomean fold of 0.8.

Real Time quantitative PCR (qRT-PCR)

Total RNA (1μg) extracted from treated and control samples were

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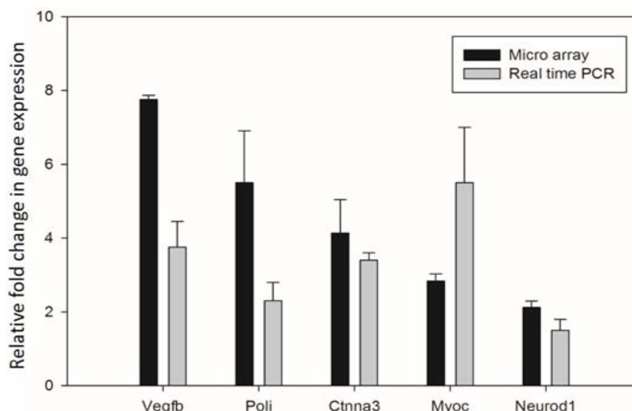

Figure 1: Validation of micro array results. Fold change in expression of five genes on 15nM rotenone treatment in VM neurons was compared between micro array analysis and qRT-PCR analysis. Values were represented in fold change and expressed in mean ±SD and n=3. Spearman's Rho correlation analysis was performed and technically a positive correlation (r=0.3) was observed.

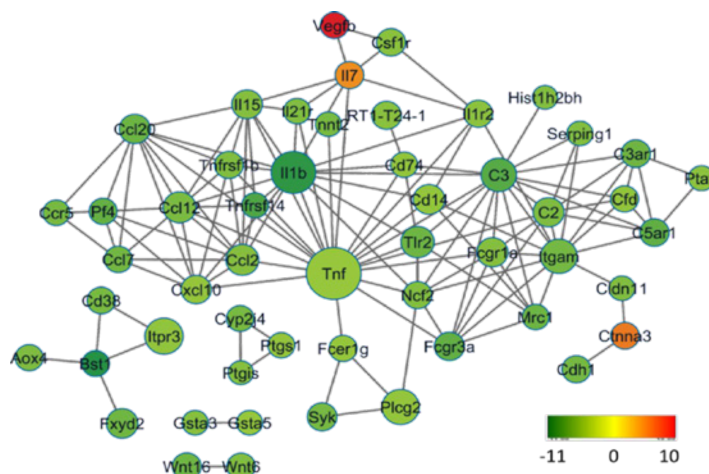

Figure 2: Gene network. Gene network for differentially expressed genes in 15nM rotenone treated VM neurons was developed using ARM. The size of the node represents the corresponding score, and the intensity of color represents the fold change expression of the gene.

reverse transcribed by Superscript III Kit (Invitrogen). Primers for the selected genes were given in Supplementary Table 1. PCR reactions were run with SYBR Green Kit (Quiagen) in ABI Prism H7500 fast thermal cycler (Applied Biosystem, CA, USA). 18srRNA was used as internal control. Amplification resulted fluorescence was analyzed and expressed in relative fold change using 2-*ΔΔ*CT method [8].

Statistics

Experiments were performed in triplicates and repeated for three times independently. Data was averaged and presented as mean ± SD. Statistical analysis for micro array data was done by student's unpaired t test. Spearman rho test were performed to find correlation between micro array data and qRT-PCR data.

Results

Micro array and validation

VM neurons at 7th DIV were treated with 15nM rotenone and incubated for 48hrs. Through micro array analysis transcriptome change in rotenone treated VM neuron was compared with DMSO alone treated VM neurons. Considering the parameters mentioned in methodology, a data with 705 up-regulated genes and 2415down-regulated genes was obtained (supplementary Table 2). In order to validate this data, five genes with different folds of expression ranging from high to low were selected randomly and their expression in VM neurons on rotenone treatment was analyzed by qRT-PCR analysis. Comparative qRT-PCR analysis in Figure 1confirmed the expression pattern of microarray for the selected genes. Most of the genes were in a positive correlation with micro array data except Myoc which had shown a higher expression in qRT-PCR than in micro array data.

Gene network

To identify key players from this large set of data, ARM method involving pathway classification and machine learning was employed. Differentially regulated genes having log fold change greater than 2 or less than 2 were selected and they were up to 240 genes. Further, their known involvement indifferent pathways were mapped using KEGG database [9]. Out of these 240 genes only 103 genes were shown to be involved in known pathways. We have used Apriori algorithm in R for mining association rules which need an input dataset consisting of transactions and a record of items in a particular transaction.

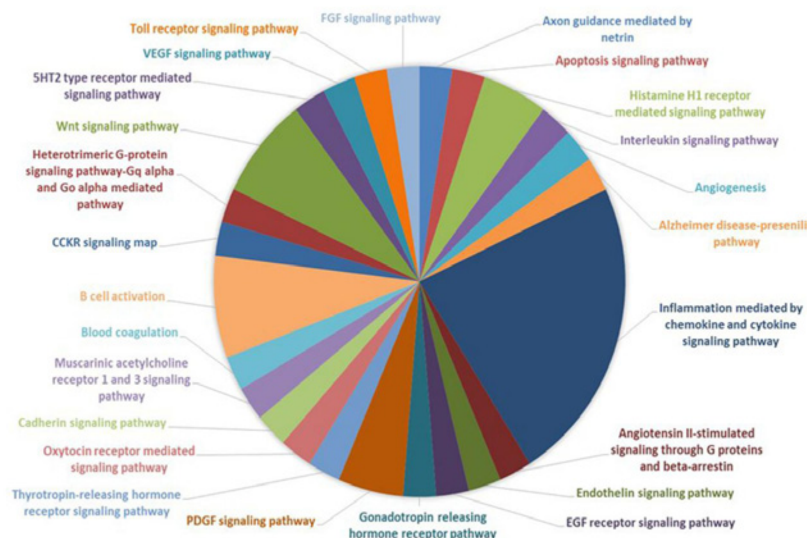

Figure 3: Pathways involved. VM neurons were treated with 15nM rotenone and ARM analysis was done to pick top 25 scored differentially expressed genes. Functional characterization of these 25 genes from the network was done using Panther database (http://www.pantherdb.org/) and corresponding pathways were depicted in a pie chart.

This algorithm is widely acceptable for transactional data and market-basket analysis [10] where frequent item sets generate strong association rule. Here we considered pathways as transactions and genes as items forming an initial dataset. The association rules obtained from this algorithm elaborates the gene relationship based purely on pathway information and was used to form the gene network. The interactions were found among 53 genes which are depicted in the form of network in Figure 2. The functional characterization of these genes was performed using Panther database [11]. A specific score was assigned to each gene node based on their interaction with other genes and their involvement in number of pathways using following formula.

S=d+p --------------------Eqation-1

where, d = degree of the node, P = number of corresponding pathways for a particular gene/node.

The top 25 scored genes from this network were functionally characterized using panther data base and was represented in form a pie chart in Figure 3. This functional analysis had shown that most of the genes belong to chemokine and cytokine-mediated inflammation and TNF was the highest scored gene among them.

Discussion

We have treated VM neurons at 7th DIV with 15nM rotenone and performed micro array analysis to analyze transcriptome change during rotenone induced toxicity. Initial data had yielded 705 up-regulated genes and 2415 down-regulated genes. Multiple-test problem arises from simultaneous measurement of expression of thousands of genes in micro array experiments. However, correcting this multiple-test problem with FWER based methods such as Bonferroni or FDR dependent methods like Benjamini-Hochberg correction reduce type 1 errors for null associations only at the expense of increasing false negative producing type II errors [12,13] and thus loosing genes with significant expression change. Therefore we opted against correction of multiple-test problem with above methods. Instead, we applied a strategy of connecting our gene expression data to their involvement in different number of significant biological pathways with ARM method. This overlapping reduces those observations made purely by chance i.e., type I errors and controls multiple-test problem to some extent. The data was further refined using association rule mining and a gene interaction network among 53 genes was developed functional characterization of the top 25 scored genes in the network was done using panther database. This functional analysis had revealed that most of the genes among them were related to inflammatory pathways. Previous studies also suggest involvement of inflammatory processes in the PD brain [14]. Several genes such as interleukin-1 beta, TNF and Toll-Like Receptors, etc. were very well known to be involved in PD [15,16]. Among all the genes present in the network, TNF had the maximum score which suggests a plausible important role in disease progression. Several studies in past have also demonstrated the significant role of TNF in neurodegeneration and PD [14,17]. TNF is usually known for its pro-apoptotic function but interestingly our data had shown a significant down regulation of TNF on rotenone treatment. This observation was supported by earlier report suggesting a role of TNF in both cell survival and cell death mechanisms [18]. Interestingly TNF receptors were known for their antagonistic functions during neurodegenerative processes with TNFR1 being pro apoptotic and TNFR2 being anti-apoptotic [19]. TNF mediated neuroprotection *via* TNFR2 was shown through persistent NFK-b activation [19]. Moreover it is to be noted that the down regulation of TNF observed here was of neuronal origin rather than glial origin as our culture was neuronal enriched culture devoid of glial cells. Interestingly our data had also shown a 2.5 fold down regulation of TNFR2 on rotenone treatment. Neurotrophic effect of proinflammatory cytokine Il1b was reported earlier [20]. The same study had also reported that neuroprotection offered by Il1b was through PI3-K/AKT mediated pro survival signaling. Neuronal Il1b was 4 fold down regulated on rotenone treatment. The other inflammation linked gene down regulated on rotenone treatment

was Itpr3 (2.1 fold), a calcium channel. Release of calcium from this receptor regulated calcium stores was shown to be regulating expression of TNF [21]. Rotenone is a mitochondrial complex-1 inhibitor and known to produce elevated ROS levels (1). Increasing evidence suggests a crosstalk between ROS and calcium channels mediated calcium signaling in neurodegeneration [22]. With these observations we hypothesize that ROS signaled- Itpr3 regulated expression of neuronal TNF might play a pro survival role in VM neurons through TNFR-2 binding with a persistent Nfkb activation *via* Nfkb/iKB kinase and/or Pkb/Akt signaling.

Conclusion

Our rotenone treated VM neuronal transcriptome analyses coupled with reported literature suggest an interesting and novel protective role of neuronal TNF in VM neurons. We suggest that elevated ROS levels on rotenone treatment might have deregulated cross talk between ROS and calcium signaling leading to down regulation of neuronal TNF and other related inflammatory molecules. This might have led to deactivation of Nfkb/iKB kinase and/or Pkb/Akt associated pro survival pathways triggering neuronal death and thus suggest one of the plausible mechanisms behind rotenone induced toxicity in VM neurons. Further experimental studies on neuronal TNF and its related pathways might prove to be a valuable therapeutic approach in treating PD.

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