Single-cell RNA Sequencing Analysis of Human Neural Grafts Revealed Unexpected Cell Type Underlying the Genetic Risk of Parkinson's Disease

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting more than 6 million patients globally. Though previous studies have proposed several disease-related molecular pathways, how cell-type specific mechanisms contribute to the pathogenesis of PD is still mostly unknown. In this study, we analyzed single-cell RNA sequencing data of human neural grafts transplanted to the midbrains of rat PD models. Specifically, we performed cell-type identification, risk gene screening, and co-expression analysis. Our results revealed the unexpected genetic risk of oligodendrocytes as well as important pathways and transcription factors in PD pathology. The study may provide an overarching framework for understanding the cell non-autonomous effects in PD, inspiring new research hypotheses and therapeutic strategies.

Keywords

Parkinson's Disease; Single-cell RNA Sequencing; Oligodendrocytes; Cell Non-autonomous; Co-expression Analysis; Transcription Factors

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1. Introduction

Parkinson's Disease (PD), the second most prevalent neurodegenerative disease, is characterized by involuntary tremors, muscle stiffness, and slow movement [1]. As of 2016, PD affected 6.1 million patients worldwide, and the number had increased by 144% since 1990 [2]. Unfortunately, the progression of PD is irreversible: patients' midbrains undergo progressive loss of dopaminergic neurons (DaNs) and accumulation of toxic α-synucleins, misfolded proteins with unclear function [3]. Though past studies have uncovered disease-associated molecular pathways, the genetic traits that control the vulnerability of individual cells in PD are yet determined [4]. Moreover, because previous research mainly focused on DaNs, the pathological significance of many different cell types remains unknown.

In the face of these challenges, single-cell RNA sequencing (scRNA-seq), a transcriptomic sampling technique, has shed new light on PD research [5]. scRNA-seq can identify cell types, reconstruct regulatory networks, and reveal the temporal transition of cell states in diverse biological processes [6]. To date, scRNA-seq has been used to study cancers [7], cardiovascular diseases [8], and neurological disorders [9]. Scientists in the PD research field have employed this technique to examine patient-derived DaNs *in vitro* [10] and dissect therapeutic neural transplants *in vivo* [11]. Nevertheless, few studies have used scRNA-seq to explore the genetic vulnerability of different cell types in PD models.

To uncover the cell-type specific mechanisms in PD pathology, we analyzed the scRNA-seq dataset of human neural grafts in PD rat models created by Tilklová et al. [11]. Through clustering, gene enrichment examination, and co-expression analysis, we discovered that DaNs and oligodendrocytes (ODCs) robustly expressed PD-related genes. Additionally, we confirmed the significance of mitochondrial and cell signaling pathways in PD pathology. Based on co-expression analysis, we identified a number of transcription factors (TFs) essential to the survival of DaNs and ODCs. Finally, we proposed a cell non-autonomous disease mechanism driven by ODCs, providing theoretical frameworks for future PD research.

2. Methods

2.1 Dataset Overview, Quality Control, and Normalization

We obtained the data from a study by Tiklová et al. [11], which was currently the only open access scRNA-seq dataset based on animal PD models. Researchers in the original study transplanted human neural grafts developed from embryonic and fetal stem cells into the striatum of rats treated with 6-hydroxydopamine, a neurotoxin to create animal PD models [12]. Six months later, they tested the prognosis of the parkinsonian rats and observed a significant improvement in their motor functions. The neural grafts were then dissected for scRNA-seq, and the dataset was deposited in Gene Expression Omnibus (GEO) [13] under the access code GSE132758.

We processed the dataset in Seurat V3, an R package for analyzing scRNA-seq data [14]. We first performed quality control to drop out genes expressed in fewer than five cells and cells with fewer than 500 RNA transcripts. We then invoked the "sctransform" function to normalize the transcript reads and select highly variable genes for downstream analysis [15].

2.2 Dimensionality Reduction, Clustering, and Cell-type Identification

Before clustering the cells, we first reduced data dimensionality using p rincipal component analysis (PCA) [6], a technique to determine sources of expression variance using highly variable genes. Only the top 15 principal components (PCs) were preserved for downstream analysis, for after PC15 not much additional variation was captured (Supplementary Figure 1). We then clustered the cells with "FindNeighbors" (shared nearest neighbors) and "FindClusters" (the Louvain method) [16]; the clusters were visualized by uniform manifold approximation and projection (UMAP) [6], a computational method for dimension reduction (Supplementary Figure 2). Eventually, we used cluster-specific markers calculated by "FindAllMarker" (non-parametric Wilcoxon rank-sum test) [17] and canonical neuronal markers curated by Abcam [18] to identify the cell type of each cluster (Supplementary Figure 3).

2.3 PD Risk Gene Enrichment Analysis

Our next goal was to investigate the expression of PD genes in different cell types. We obtained 20 familial PD genes from the database of Online Mendelian Inheritance in Man, a free-access database of human genetic traits [19]. The cell-type-specific enrichment levels of these genes were visualized in ridge plots. We also evaluated the expression of over 2000 PD risk genes gathered from three sources: DisGeNET [20], ParkinsonsUK-UCL [21], and a study by Reynolds et al [22]. The genes were identified by genome-wide association studies (GWAS), an approach to associate genetic variants with disease phenotypes [23].

To analyze the PD risk genes, we used Expression Weighted Cell Type Enrichment (EWCE), a technique to determine the enrichment significance of a gene list in a cell type [24]. EWCE accepted a target gene list and a background list as inputs. In our case, the target list comprised PD risk genes, and the background list was chosen randomly from all genes expressed in the dataset. The two lists were processed in a series of steps: 1) the average expression levels of every gene in the background list were calculated in a given cell type; 2) the previous step was repeated in the target list; 3) the average expression of the background list was used to compute the probability distribution of expression in the cell type 4) the probability distribution was used to calculate the standard deviation of expression in the target list. Ultimately, a high standard deviation indicated that the target list was significantly expressed in the cell type.

2.4 Co-expression Analysis

To further investigate the genetic traits of different cell types, we employed Co-Expression Modules identification Tools (CEMiTool), a computational approach to discover co-activated gene modules [25]. We first subset the dataset to focus on relevant cell types identified by PD risk gene screening. We then invoked "cemitool" and uncovered two co-expression gene modules. To understand the functional characteristics of the modules, we performed gene ontology (GO) analysis using g:Profiler, an online server for GO analysis [26] (Supplementary Figure 4).

We also sought to explore the regulatory networks of each module. We utilized iRegulon, an algorithm for identifying the motifs and TFs of a gene list by the ranking-and-recovery methods [27]. During the ranking step, each gene in the co-expression module was scanned for its enriched motifs and TFs. After all genes were scanned, one list of motifs and one list of TFs were created. The algorithm then ranked the motifs and TFs according to the number of genes they were associated with. During the recovery step, the genes associated with the ranked lists of motifs/TFs were compared with a randomly generated background list of genes. Eventually, the algorithm recognized genes that successfully distinguished the ranked list from the unranked background list as candidate target genes.

3. Results

Our research was guided by two questions: 1. how are PD related genes expressed in different cell types in the brain? 2. what can the transcriptomic profiles of individual cells suggest about their vulnerability in PD? To address these questions, we analyzed scRNA-seq data of human neural grafts transplanted to the midbrain of PD rat models. Curiously, the results revealed the enrichment of PD-related genes in not only DaNs but also ODCs. Further analysis of co-expressed genes suggested that DaNs and ODCs interact via myelination and paracrine signaling, which may render new insights into PD pathogenesis.

3.1 Clustering and Marker Testing Identified Neural Lineage Cells

Our fundamental goal was to identify the cellular composition in the neural grafts. To start with, we first filtered out low quality cells and normalized the molecular counts. Next, we reduced the dimensions of the data and clustered the cells by various algorithms [6] (Supplementary Figures 1 and 2). To determine the cellular identities in the samples, we carefully examined the top three computational markers of each cluster and the expression of canonical cell markers [18] (Supplementary Figure 3) (Figure 1).

In the end, seven cell types were uncovered: TH+ immature neurons (TH+ IMNs), TH+ postmitotic neurons (TH+ PMNs), astrocytes (ASTs), oligodendrocytes (ODCs), radial glia-like cells (RGL-LCs), cancer-like stem cells (CLSCs), and neural crest-derived stem cells (NC-DSCs)

(Figure 2). Notably, TH+ PMNs closely resembled DaNs due to their expression of TH (DaN marker), NeuN, and SYP (mature neuronal markers). ASTs were characterized by astrocyte markers AQP4 and GFAP; ODCs were identified by oligodendrocyte markers OLIG1, OLIG2, and MBP. Overall, the clustering and cell type assignment results confirmed the presence of neural lineage cells—especially DaN-like cells—in the neural grafts, which provided foundation for downstream analysis (Figure 2).

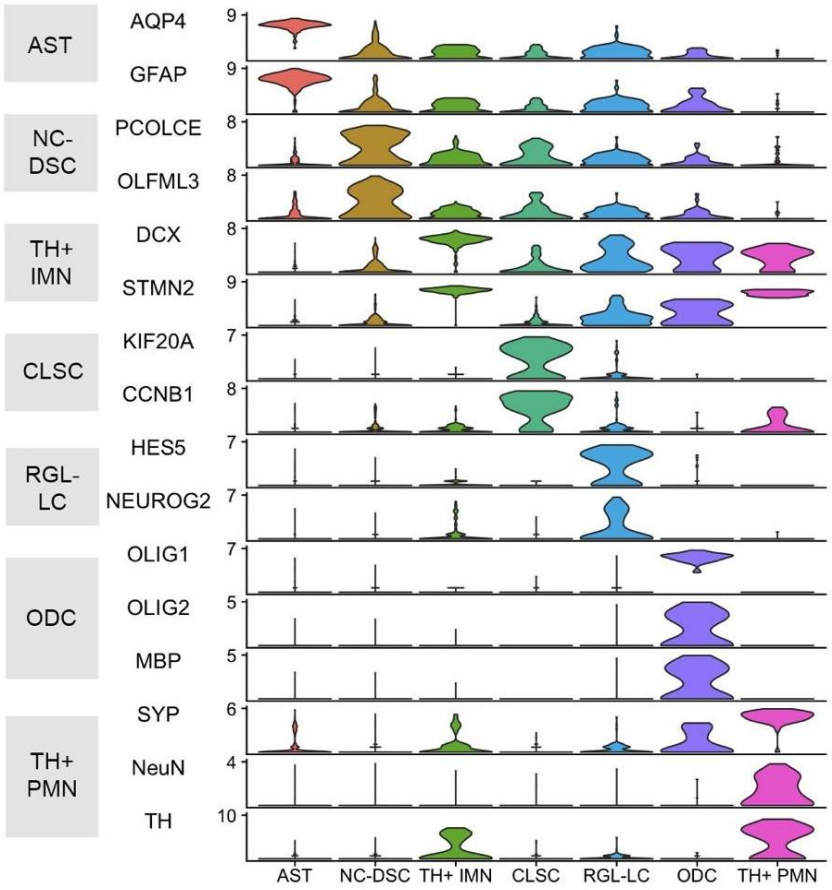


Figure 1: The Expression of Marker Genes Across Seven Cell Types. *In the stacked violin plots, the height of the "violin" is the enrichment level of a gene, and its width is the relative proportion of the cells at an enrichment level. The expression of marker genes revealed seven unique cell types in the dataset: TH+ immature neurons (TH+ IMNs), TH+ postmitotic neurons (TH+ PMNs), astrocytes (ASTs), oligodendrocytes (ODCs), radial glia-like cells (RGL-LCs), cancer-like stem cells (CLSCs), and neural crest-derived stem cells (NC-DSCs).*

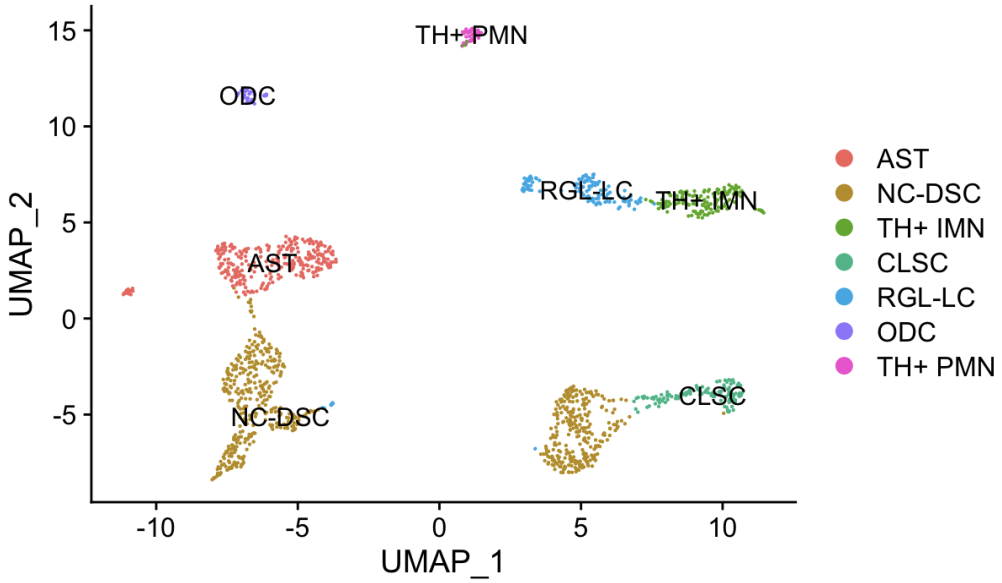


Figure 2: Cell Clusters with Assigned Identities Visualized by UMAP. *The seven cell types were visualized by uniform manifold approximation and projection (UMAP), a dimension reduction algorithm. UMAP_1 and UMAP_2 represented the first two of the fifteen dimensions of the dataset.*

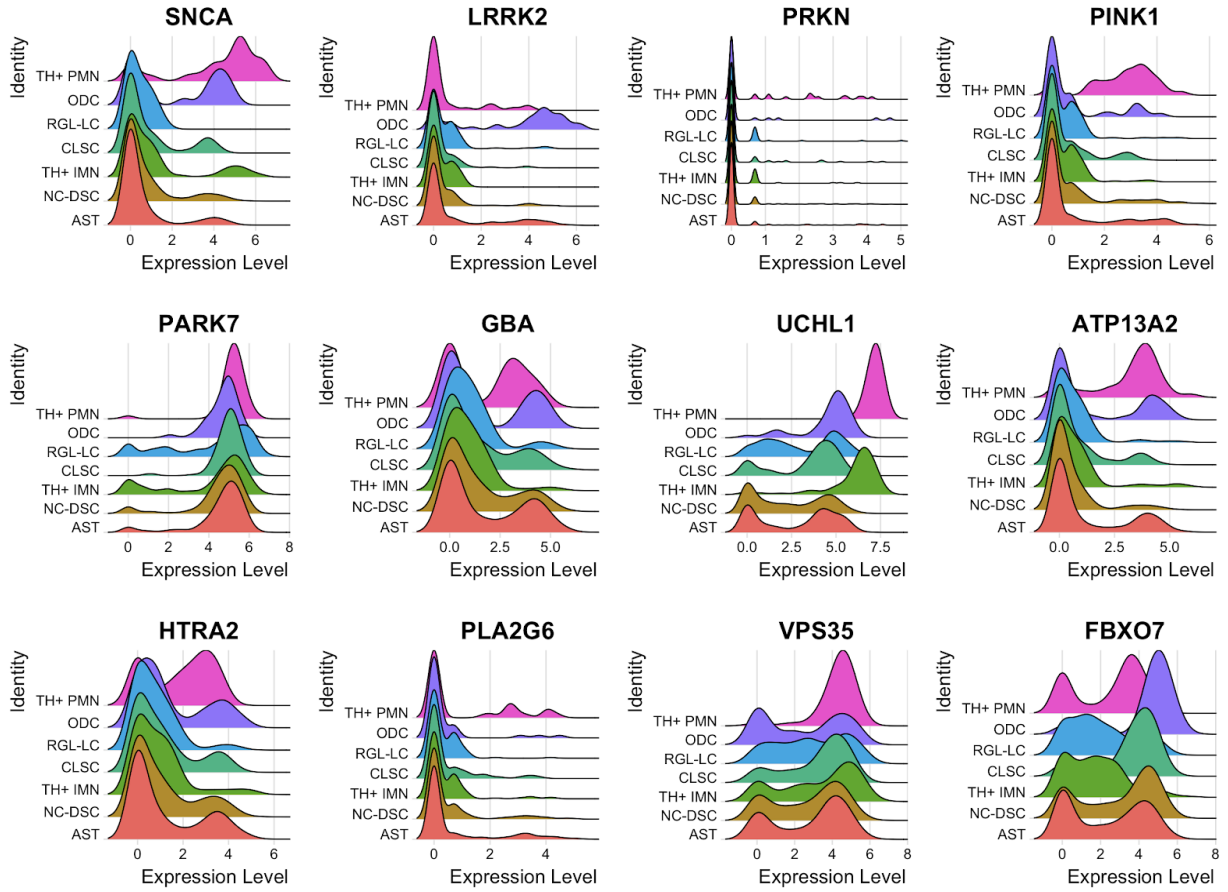
3.2. Gene Enrichment Analysis Revealed the Association of TH+ PMNs and ODCs with PD Pathology

After determining the identity of each cluster, we attempted to answer the first guiding question: how are PD related genes expressed in different cell types in the brain? Specifically, we aimed to explore genes associated with both familial and sporadic forms of PD. The familial cases of PD could be caused by mutations in SNCA, LRRK2, PINK1, PARK7, etc. [28], while the genetic cause for sporadic PD was more obscure. Luckily, genome-wide association studies (GWASs)—a method to relate genetic mutations to disease phenotypes—still identified commonly mutated risk genes among sporadic PD patients [23] [29]. In this section, we sought to evaluate the expression of familial PD genes and GWAS-identified risk genes across all clusters, which may reveal the genetic risk in each cell type.

3.2.1 Screening Familial PD Genes Across Seven Cell Types

We found 20 genes associated with familial cases of PD from Online Mendelian Inheritance in Men (OMIM), a free access database of human genetic traits. The expressions of these genes were visualized in ridge plots (Figure 3). Not surprisingly, TH+ PMNs had an above-average expression of ten familial PD genes (SNCA, PINK1, UCHL1, ATP13A2, HTRA2, VPS35, EIF4G1, GBA2, DNAJC6, and SYNJ1), which corresponded to the fact that DaNs tend to develop PD phenotypes [30]. Second to TH+ PMNs, ODCs showed an elevated expression of five genes (LRRK2, SNCA, FBXO7, GIGYF2, and YPS13C). In particular, LRRK2, the gene underlying the autosomal

dominant Parkinson disease 8, was uniquely expressed in ODCs but not in TH+ PMNs [31]. Based on this observation, we wondered whether the mutations of LRRK2 may cause the malfunctioning ODCs to influence DaNs through cell-cell interaction (the cell non-autonomous effects of ODCs). Finally, no other cell types demonstrated a significantly high expression of familial PD genes, so the results mostly reflected the vulnerability of TH+ PMNs and the potential involvement of ODCs in PD.



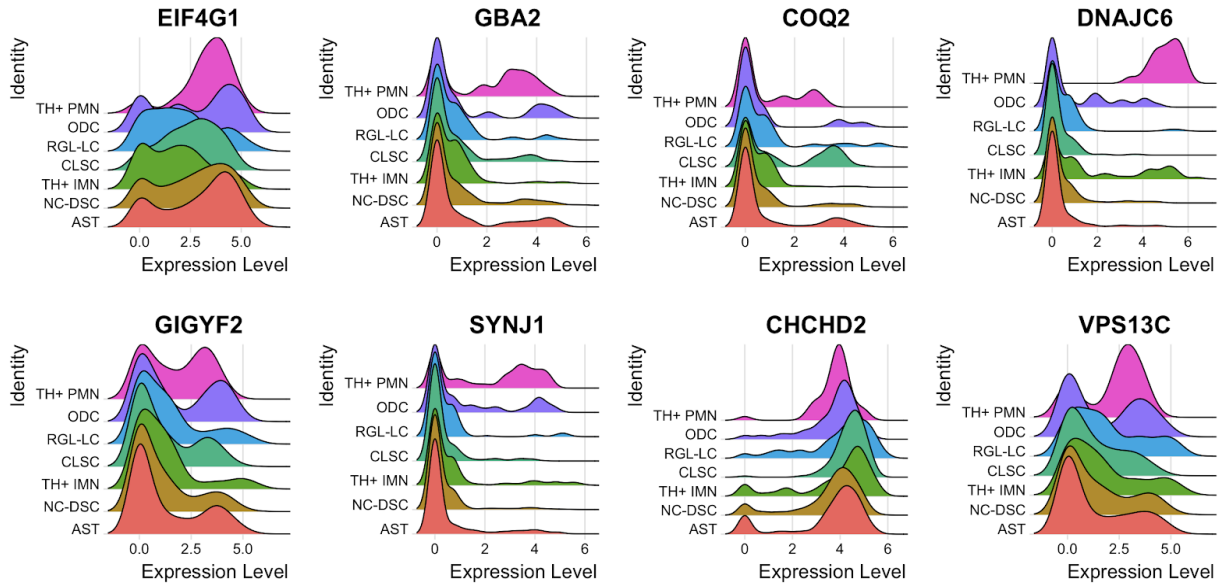


Figure 3: Expression Pattern of Familial PD Genes Visualized by Ridge Plots. *The horizontal position of the "ridge" is the expression level of a gene, and its height is the relative proportion of the cells at an expression level. TH+ PMNs had an above-average expression of ten genes: SNCA, PINK1, UCHL1, ATP13A2, HTRA2, VPS35, EIF4G1, GBA2, DNAJC6, SYNJ1, and VPS13C. ODCs had above-average expression of five genes: LRRK2, SNCA, FBXO7, GIGYF2, and YPS13C. No other cell types demonstrated a significantly elevated expression of familial PD genes.*

3.2.2 Screening PD Risk Genes Identified By GWAS

To offset the potential bias from a small number of familial PD genes, we next investigated the expression of more than 2000 risk genes identified by GWAS. We gathered the risk genes from three separate sources: DisGeNET [26], ParkinsonsUK-UCL [21], and a study by Reynolds et al. [22]. The three gene sets each had 2077, 330, and 88 items, and we analyzed them using Expression Weighted Cell Type Enrichment (EWCE), a technique to calculate the enrichment significance of a gene list [24]. In fact, the ability of EWCE to examine a list instead of one gene at a time greatly improves the evaluation efficiency.

In the first two lists (DisGeNet and ParkinsonsUK-UCL), TH+ PMNs had the highest enrichment significance, and ODCs had the second highest. In the third list (Reynolds et al.), however, only ODCs had a significant expression. This unexpected outcome might result from the small number of genes in the list (only 88 genes). Overall, the screening of over 2000 PD risk genes confirmed that both TH+ PMNs and ODCs were genetically relevant to PD.

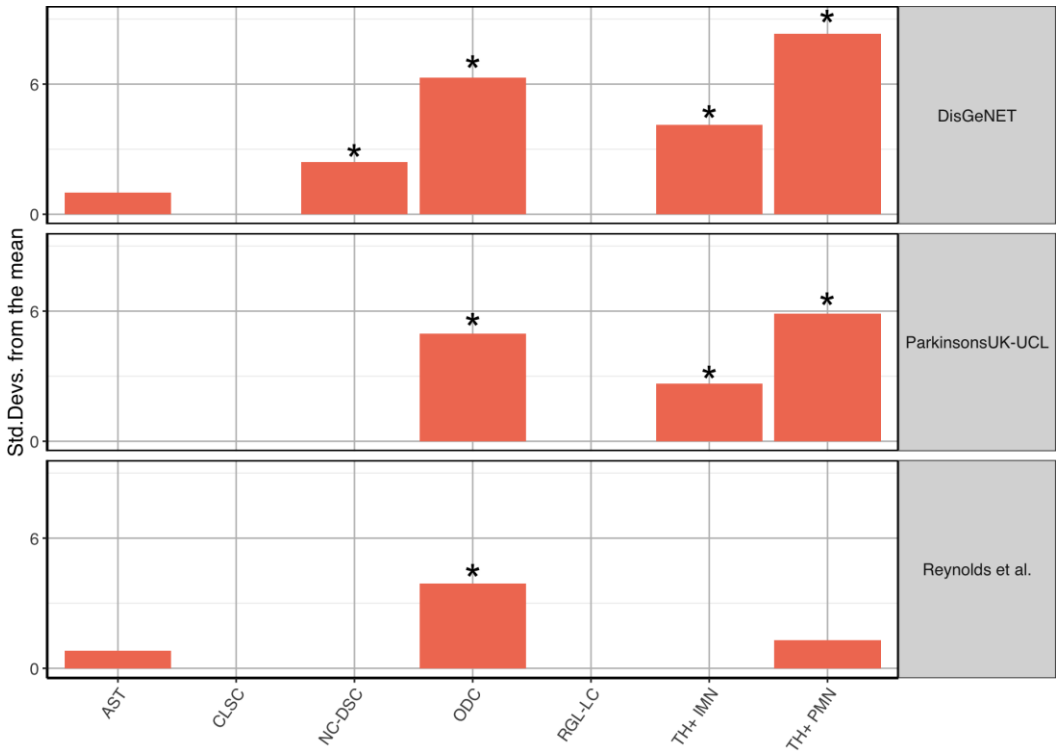


Figure 4: Enrichment Analysis of PD Risk Genes. *A high standard deviation suggested that the target list was significantly expressed in the cell type. The asterisk (*) indicated that the expression level was statistically significant ($p<0.05$). In general, PD risk genes were enriched in both TH+ PMNs and ODCs.*

3.3 Gene Co-expression Analysis Uncovered PD-related Pathways and Transcription Factors in TH+ PMNs and ODCs

We next sought to answer our second guiding inquiry: what can the transcriptomic profiles of cells suggest about their vulnerability in PD? More specifically, we aimed to identify genes whose expression rises and falls together in TH+ PMNs and ODCs. By elucidating the functions of coactivated genes, we may discover molecular pathways and TFs that determine the cell-type specific risk to PD.

We identified co-activated genes with Co-Expression Modules identification Tool (CEMiTool), a computational approach to cluster genes based on their expression pattern [25]. Eventually, two co-expression modules with 301 and 75 items were discovered. Module 1 (M1) was enriched in TH+ PMNs, while module 2 (M2) was overrepresented in ODCs (Figure 5). Though we were also curious about genes expressed in both cell types, we did not find a module that was enriched in both.

After determining the co-activated genes, we planned to examine the gene ontology (GO) and TFs of each module using g:Profiler [26] and iRegulon [27]. g:Profiler is an online tool to annotate the

biological functions of a gene set, and iRegulon is a method to detect TFs that regulate genes in a target list. The results for M1 and M2 were described separately below.

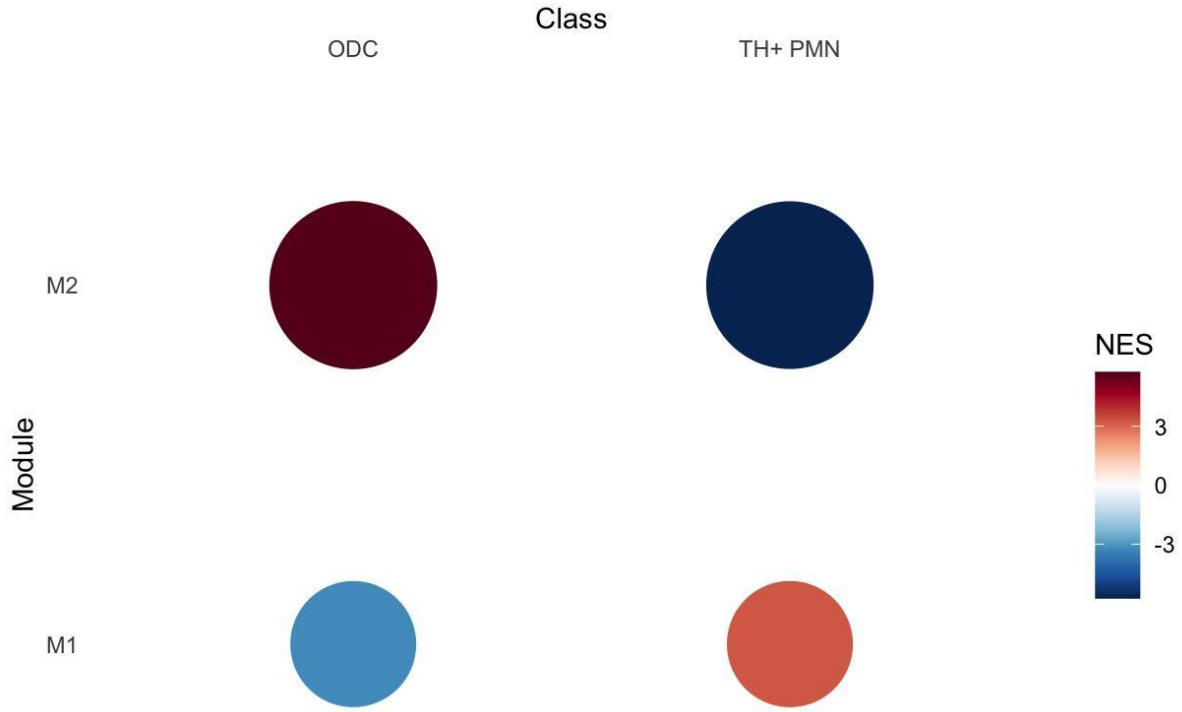


Figure 5: Co-expression Module Enrichment Levels in ODCs and TH+ PMNs. *The sizes and colors of the dots represented the enrichment degree of a gene module in a cell type. A redder and larger dot had a higher enrichment degree. M1 was mostly enriched in TH+ PMNs, while M2 was overrepresented in ODC.*

3.3.1 Gene Ontology and Transcription Factor Analysis of Module 1

GO analysis demonstrated that M1 was strongly associated with the term "Parkinson's Disease". In fact, the GO term contained 48 out of 301 genes from M1, including those related to familial PD (SNCA, PARK7, UCHL1, etc.), ATP synthetase (ATP5F1A, ATP5F1B, etc.), and cytochrome c oxidase (COX5B, COX7C, etc). Not surprisingly, M1 also had mitochondria-associated terms such as "oxidative phosphorylation" and "ATP-synthesis coupled electron transport", which supported the theory of mitochondrial stress in PD [32]. Furthermore, the GO terms included "vesicles", "extracellular exosome", and "proton transmembrane transport", reinforcing the fact that the signaling capacity of DaNs is impaired in PD [33]. Overall, the GO analysis of M1 confirmed the association of PD pathology with the mitochondrial and cell signaling pathways.

iRegulon uncovered fifteen TFs for M1, and five of them had known association with PD (TAF1, REST, NFE2, UBE2K, and HSF1) (Supplementary Table 1). Interestingly, literature search suggested that all five TFs were neuroprotective. For example, RE1-silencing transcription factor (REST), a TF that suppresses the expression of neuronal genes outside neural-lineage cells, was found to be significantly downregulated in patient-derived DaNs [34]. The ablation of REST in

mice [35] also increased the animals' susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a drug used to create PD models [36]. Furthermore, heat shock factor 1 (HSF1), a gene that facilitates the correct folding and distribution of proteins, was observed to reduce the concentration and toxicity of α-synuclein aggregates [37]. Based on these facts, researchers may consider the five TFs of M1 as therapeutic targets to promote the survival of DaNs in PD patients' midbrain.

Out of the remaining ten TFs, we noted two for their profound influence in the central nervous system (CNS). Kruppel-like factor 4 (KLF4), a gene involved in a wide range of cellular responses, could suppress the regenerative capacity of neurons in the CNS [38]. Meanwhile, tumor protein p53 (TP53), a famous tumor suppressor, would protect neurons from death when it was ablated [39]. Though no existing studies linked KLF4 and TP53 to PD, the two TFs may be generally involved in the processes of neural regeneration and degeneration. Therefore, future researchers may also explore the potential implications of KLF4 and TP53 in PD therapies.

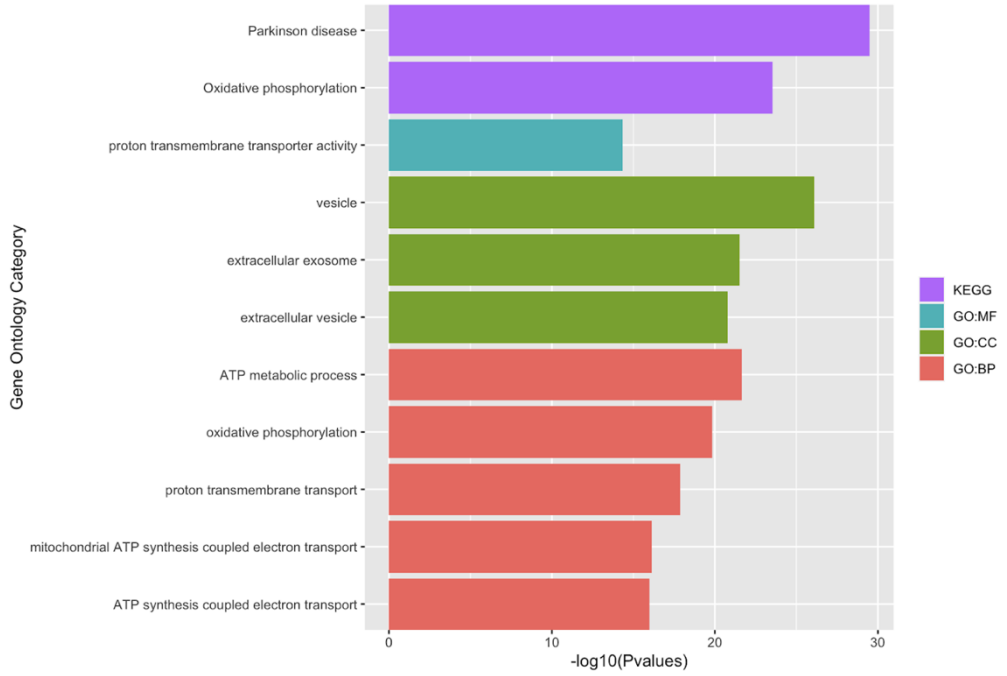


Figure 6: GO Analysis of M1 genes. *The length of the bar indicated the confident level of each GO term, and a longer bar represented more confidence. The GO analysis of M1 demonstrated that PD genetics were related to both mitochondrial pathways and cell signaling.*

3.3.2 Gene Ontology and Transcription Factor Analysis of M2

The GO terms of M2 included "nervous system development", "neurogenesis", and "myelin sheath", which generally described the supportive functions of ODC—cells where M2 was most enriched. Though the GO results of M2 did not include "Parkinson's Disease", the terms "synapse"

and “cell junction” hinted at the cell non-autonomous mechanisms of PD. We thus hypothesized that ODCs may interact with DaNs through synaptic transmission and cell junctions, potentially influencing the susceptibility of DaNs.

M2 also had two PD-related TFs: HSF1 and Serum response factor (SRF) (Supplementary Table 2). SRF, a gene that regulates cytoskeleton growth, is important for both DaNs and ODCs [40]. For instance, knocking out SRF in DaNs can increase the neuronal susceptibility to MPTP [41], while silencing it in neural precursor cells may inhibit oligodendrocyte differentiation [42]. Notably, a past study reported that SRF-deficient neurons could affect healthy ODCs through paracrine signaling [43], so we wondered whether the downregulation of SRF in ODCs may likewise increase the vulnerability of DaNs. In general, SRF serves a neuroprotective role in DaNs and ODCs, and its significance in the cell non-autonomous mechanisms of PD should be further examined.

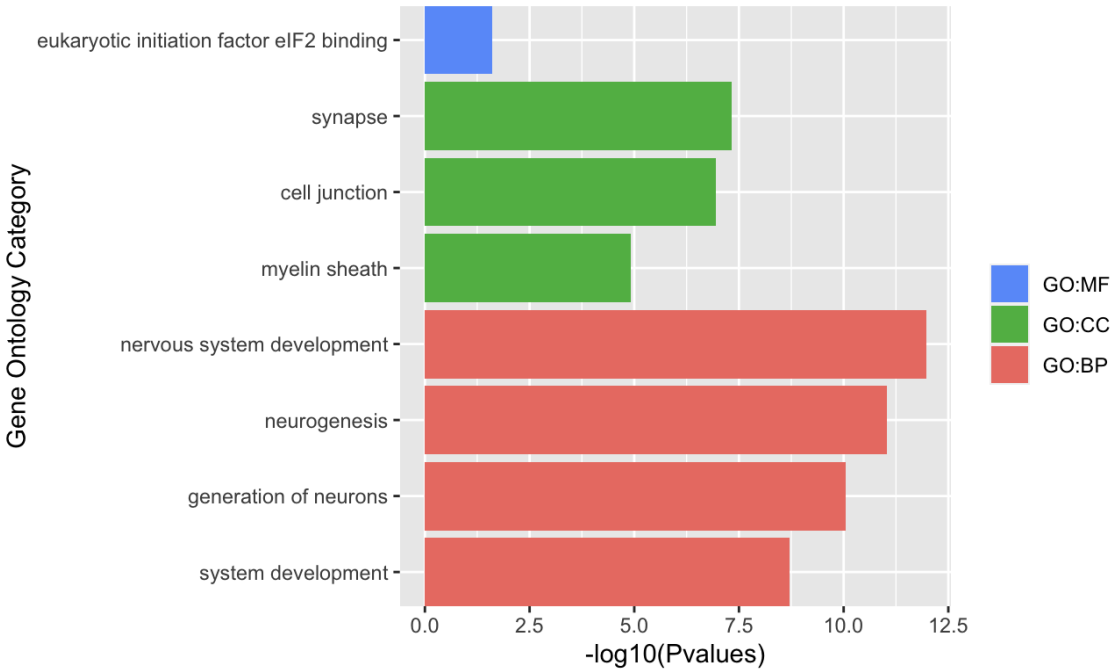


Figure 7: GO Analysis of M2 genes. *The bar plot demonstrated GO terms related to the functions of ODCs. Though the GO terms did not include “Parkinson’s Disease”, there were “synapse” and “cell junction”, providing clues for cell non-autonomous effects; ODCs may interact with DaNs through synapse or cell junctions, thereby influencing the phenotypes of DaNs in PD.*

4. Discussion

Overall, our computational analysis revealed that PD was not only associated with DaNs but also ODCs. Further investigation of co-activated gene modules indicated that DaNs may be impacted by both cell-autonomous (mitochondrial functions) and non-autonomous effects (exosomes,

paracrine, etc.). In particular, ODCs might affect DaNs through myelination, cell junctions, and synaptic transmission. We also noted that the loss of neuroprotective genes such as REST, HSF1, and SRF might exacerbate individual cells' susceptibility to PD.

Admittedly, our study also has some shortcomings. Because scRNA-seq is a relatively new technique, few scientists in the PD research field have utilized it in their studies. In fact, the scRNA-seq dataset we use is the only open access one that includes animal PD models, and it inevitably has some limitations. For instance, the dataset does not contain samples from wild type rats, so we could not determine the downregulation and upregulation of genes in the PD rat models. In addition, the number of TH+ PMNs and ODCs in the neural grafts is relatively small, which might have introduced bias into our study. Increasing sample size is also challenging, for integrating multiple datasets always creates more caveats.

To address these problems, we propose a scRNA-seq study that would include samples from wildtype rats and a large number of mature neural cells. Moreover, we would test our hypothesis that the silencing of SRF (a TF described in section 3.3.2) in ODCs would induce phenotypic changes in wildtype DaNs. To achieve the goal, we would perform Cre-dependent ablation of SRF in ODCs only. We would monitor myelination states, paracrine signaling, and signs of cellular stress responses in DaNs to verify the cell non-autonomous effects of ODCs. Eventually, we wish to elucidate the functional interdependence between ODCs and DaNs, thereby illuminating the genetic and cellular basis of PD pathogenesis.

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6. Conflict of Interest

The authors declare no conflict of interest.

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Declaration of Academic Integrity

I hereby confirm that:

1) I have submitted complete and correct information on the composition of the research team and supervising teacher.
2) The present paper is based on research carried out under the guidance of the supervising teacher.
3) The present paper does not contain any published or written work of others, other than those that are acknowledged and cited, and it is not involve plagiarism or any other academic dishonesty.

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