Gene Expression Profiling of Post Mortem midbrain of Parkinson's Disease patients and Healthy Controls

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Article

Gene Expression Profiling of *Post Mortem* Midbrain of Parkinson's Disease Patients and Healthy Controls

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Abstract: Parkinson's disease (PD) stands as the most prevalent degenerative movement disorder, characterized by the loss of dopaminergic neurons in the substantia nigra of the midbrain. In this study, we assessed the transcriptome by analyzing post-mortem mRNA extracted from the substantia nigra of individuals with PD and healthy controls. A total of 16,148 transcripts were identified, with 92 mRNAs displaying differential expression between PD and control groups. Specifically, 33 mRNAs were significantly upregulated, while 59 mRNAs were downregulated in PD compared to controls. The identification of statistically significant signaling pathways, with an adjusted p-value threshold of 0.05, unveiled noteworthy insights. Particularly, enriched categories included cardiac muscle contraction (involving genes such as ATPase Na+/K+ transporting subunit beta 2 (ATP1B2), solute carrier family 8 member A1 (SLC8A1), and cytochrome c oxidase subunit II (COX2)), GABAergic synapse (involving GABA type A receptor-associated protein like 1 (GABARAPL1), G protein subunit beta 5 (GNB5), and solute carrier family 38 member 2 (SLC38A2)), autophagy (involving GABARAPL1 and tumor protein p53-inducible nuclear protein 2 (TP53INP2)), and Fc gamma R-mediated phagocytosis (involving amphiphysin (AMPH)). These findings uncover new pathophysiological dimensions underlying PD, including the involvement of cardiac muscle contraction and specific mitochondrial activity. This knowledge not only contributes to better diagnostic precision but also paves the way for the development of new targeted therapies.

Keywords: mRNAs; RNA sequencing; Parkinson's disease; transcriptome analysis; substantia nigra

1. Introduction

Parkinson's disease (PD) stands out as the most prevalent movement disorder and neurodegenerative disease after Alzheimer's dementia, affecting approximately seven million people globally [1,2]. Clinically, PD is a heterogeneous condition primarily characterized by resting tremor, bradykinesia, and rigidity. Additionally, numerous non-motor symptoms, equally debilitating and often preceding motor manifestations, contribute to the complexity of the disease [3–6]. Despite considerable research efforts, encompassing both preclinical and clinical studies, PD remains incurable. The progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), a crucial part of the midbrain regulating movement tone and velocity, persists without effective intervention, and the complete pathomechanisms behind this degeneration remain incompletely elucidated [1,7].

Pathophysiologically, PD has a multifactorial origin involving complex interactions between various genetic and environmental factors. Some factors affect the elderly in general, while others

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seem more specifically tied to this disorder. It's noteworthy that post-mortem studies consistently report that over 60% of DA neurons in the SNc are already degenerated when overt clinical signs manifest. This indicates that PD is neuropathologically evident long before its clinical onset, suggesting a period during which the human brain can compensate for dopaminergic loss until reaching a "clinical threshold" for PD [8–10].

Histological studies reveal that PD is characterized by the abnormal deposition of the insoluble alpha-synuclein protein, forming aggregates known as Lewy bodies [11]. These protein aggregates progressively accumulate throughout the brainstem and various neocortical and limbic regions, reflecting the progressive degeneration of the entire central nervous system (CNS) [12]. More recently, a neuroinflammatory state has been observed in the brains of PD patients, particularly evident in the SNc [13,14]. Both neuroinflammation and dysfunctional activation of the immune system within the CNS significantly contribute to PD pathology and pathophysiology [15]. The inflammasome, a crucial complex of immune-modulating receptors and sensors, plays a role in recruiting proteins associated with apoptotic mechanisms through caspase-1 activation [16,17]. Caspase-1, in turn, activates the pro-inflammatory cytokines interleukin (IL)-1β and IL-18, perpetuating the neuroinflammatory state in PD brains [17,19–21]. A recent proposed model suggests that alpha-synuclein activates the inflammasome in the SN, leading to IL-activated pro-inflammatory profiles, neuronal death, and clinical symptoms [22–24].

Genetically, several genes or gene variants, including leucine-rich repeat kinase 2 (LRRK2), synuclein alpha (SNCA), glucosylceramidase beta-1 (GBA1), Parkin RBR E3 ubiquitin protein ligase (PARKIN), and PTEN-induced kinase 1 (PINK1), have been implicated in causing PD [25–28]. Molecular profiling studies of post-mortem SNpc samples, aimed at identifying differential molecular expression changes specific to PD compared to controls, have been conducted [29]. For instance, Simunovic et al. [30] used RNA microarrays to analyze SNpc gene expression in PD samples, identifying dysregulation of known molecular regulatory pathways in PD, including dysfunction in mitochondrial and oxidative stress-induced cellular responses [31,32]. In a recent comparative gene expression analysis on laser-dissected neurons from SNpc, Zaccaria et al. [33] revealed 52 dysregulated genes in PD samples compared to controls.

In our study, we conducted mRNA analysis and subsequent enrichment using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) to assess mRNAs extracted postmortem from the SN of subjects with PD and healthy controls.

2. Results

In the examination of mRNA deregulation in the SNc of PD patients, we conducted gene expression profiling on 8 post-mortem SNpc samples from PD patients and 6 from healthy control subjects (CTRL) using next-generation sequencing (RNA-Seq). The aim was to identify specific and differential changes in molecular expression. Following the removal of low-quality reads and adapter sequences, the high-quality reads were aligned against the human genome reference (hg38). In detail, we identified a total of 16,148 transcripts (Supplementary Table 1, sheet A), with 92 mRNAs differentially expressed (DEGs) between the two groups (PD vs. CTRL). Among these, 33 mRNAs were significantly upregulated (Table 1; Figure 1), while 59 mRNAs were significantly downregulated in PD compared to CTRL (Table 2; Figure 1). The normalized count of mRNAs is available at ArrayExpress (E-MTAB-13295).

The heatmap (Figure 1A) illustrates statistically significant differences in mRNA expression profiles between PD and CTRL. The volcano plot (Figure 1B) depicts the distribution of differentially expressed transcripts by their fold change and p-values. The most upregulated genes are toward the right, the most downregulated toward the left, and the most statistically significant genes at the top.

Table 1. mRNAs down-expressed in PD subjects compared to controls (padj ≤0.05 and |FC| ≥1.5).

Gene ID	Fold change	Gene ID	Fold change
ETNPP1	-7.435	AC093330.1	-2.718
MIND4P12	-5.475	MAP4	-2.693

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CP	-5.444	S1C1A2	-2.651
M1C1	-5.02	TXNIP	-2.625
PPDPF	-4.631	AGf	-2.61
PAQR6	-4.469	RHOBTB3	-2.604
ACBD7	-4.345	CST3	-2.562
MOBP	-4.315	SPARC11	-2.55
AQP1	-4.069	MIND2P28	-2.544
TAG1N	-4.001	HIPK2	-2.427
MBP	-3.977	ZEB2	-2.425
PAJP2B	-3.892	NDRG2	-2.408
PPP1R1B	-3.766	MIURN	-2.382
MTATP6P1	-3.736	DAAM2	-2.381
MT-C02	-3.5	NFIX	-2.343
E1OV17	-3.456	FADS2	-2.325
SCARA3	-3.419	ATP1B2	-2.268
MT-C03	-3.338	HEPACAM	-2.263
TP53INP2	-3.336	SHIN1	-2.203
FAM107A	-3.168	FAR1	-2.182
SEPTIN4	-3.158	AHCYU	-2.157
A1AD	-3.122	EPAS1	-2.075
MT-CO1	-3.116	PH1DB1	-2.058
IRAG1	-3.089	GFAP	-2.043
P1AAT3	-3.056	MAP4K4	-2.041
MT-CYB	-3.045	PAD12	-1.938
CINNA3	-2.949	CNP	-1.916
FAT3	-2.906	PKP4	-1.874
BCAS1	-2.857	WNK1	-1.714
TSC2204	-2.767		

Table 2. mRNAs over-expressed in PD subjects compared to controls (padj ≤0.05 and |FC| ≥1.5).

Gene ID	Fold change	Gene ID	Fold change
I110RA	12.026	FYB1	4.204
AMPH	9.664	ARHGDIB	4.111
HS6ST3	8.405	S1C38A2	4.104
VSN11	7.195	GNB5	3.998
CO1GA1T1	6.373	NAA30	3.930
PRDM11	6.234	S1C8A1	3.409
11CAM	6.208	DYNLL1	2.680
GPR34	6.141	HSPH1	2.666
ZNF618	5.543	SPP1	2.660
RCSD1	5.419	BSN	2.518
CRCP	5.257	GABARAP11	2.471
1NA	4.831	YWHAG	2.443
PIPRT	4.812	QDPR	2.060
GUCY1B1	4.422	DNAjC5	2.042
S1CSA3	4.338	NEAT1	1.926
SRGN	4.250	TNPO1	1.890
SCN8A	4.236		

We employed the pathfindR tool to analyze significant DEGs in PD based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Enrichment analysis was performed to explore functional variations between the two groups and investigate pathways

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potentially associated with PD. Statistically significant signaling pathways included (hsa04260) cardiac muscle contraction, (hsa04727) GABAergic synapse, (hsa04140) Autophagy, and (hsa04666) Fc gamma R-mediated phagocytosis (Figure 2A and Supplementary Table 1).

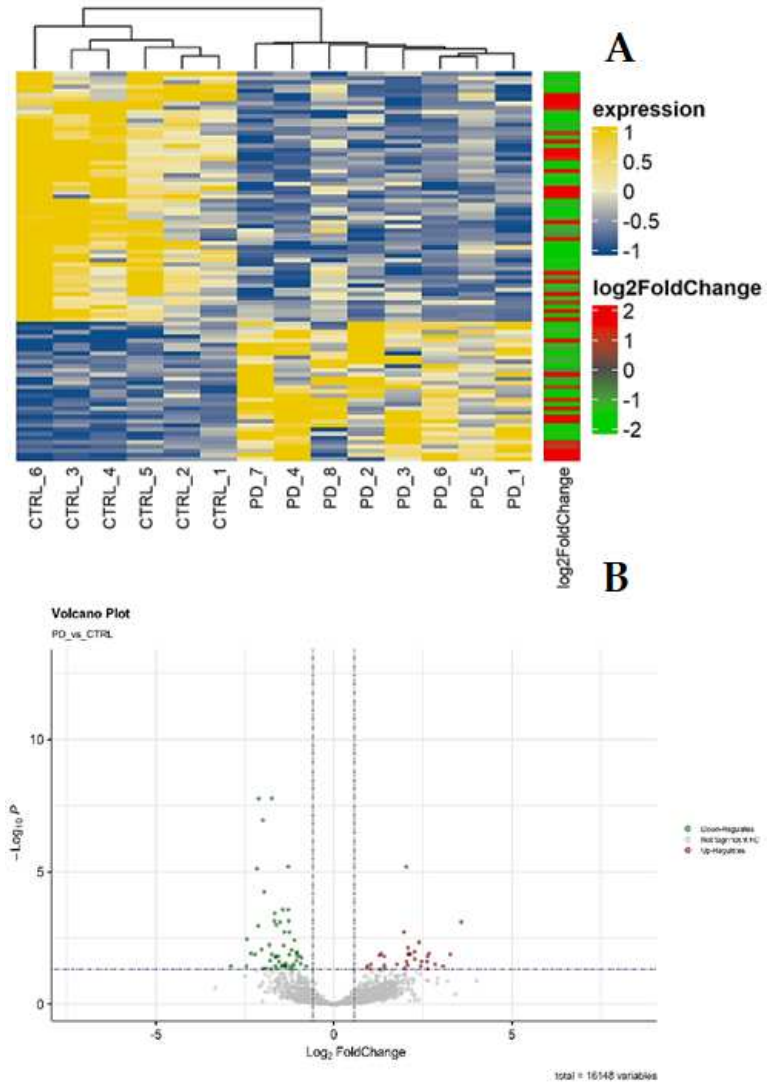

Figure 1. Visualization of differentially expressed genes (DEGs). (A) Heatmap of significant DEGs in patients with Parkinson's disease (PD) and healthy controls (CTRL). In yellow, the genes with up normalized expression level, whereas in blue the down genes. The log2 (foldChange) bar indicates in red and in green the up and down-regulated genes, respectively. (B) Volcano plot of significant DEGs based on fold changes and p-values. The green color shows the down-regulates genes, whereas the red color shows the up-regulates genes.

For Gene Ontology (GO) enrichment, we identified both down-regulated and up-regulated genes involved in various Molecular Functions or Biological Processes (Figure 2B and Supplementary Table 2). Notable enrichments encompassed chemical synaptic transmission, cellular response to hypoxia, autophagosome assembly, GABA receptor binding, and cell communication by electrical coupling in both heart and brain activity.

To explore Diseases and Biological Functions significantly enriched in DEGs and assess potential associations with PD susceptibility in the SNc, we employed Ingenuity Pathway Analysis (IPA). The analysis revealed significant enrichment in Neurological Disease, with multiple annotations related to disorders of basal ganglia, movement disorders, neuromuscular disease, dyskinesia, progressive motor neuropathy, familial neurological disorder, Parkinson's disease, progressive neurological disorder, abnormal morphology of the nervous system, and tauopathy (Figure 3 and Supplementary

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Table 3). The DEGs IPA Network Analysis identified 7 networks with nodes and interactions associated with the top Diseases or Functions Annotations, such as Neurological Disease, Organismal Injury and Abnormalities, and Psychological Disorders (Figure 4 and Table 3).

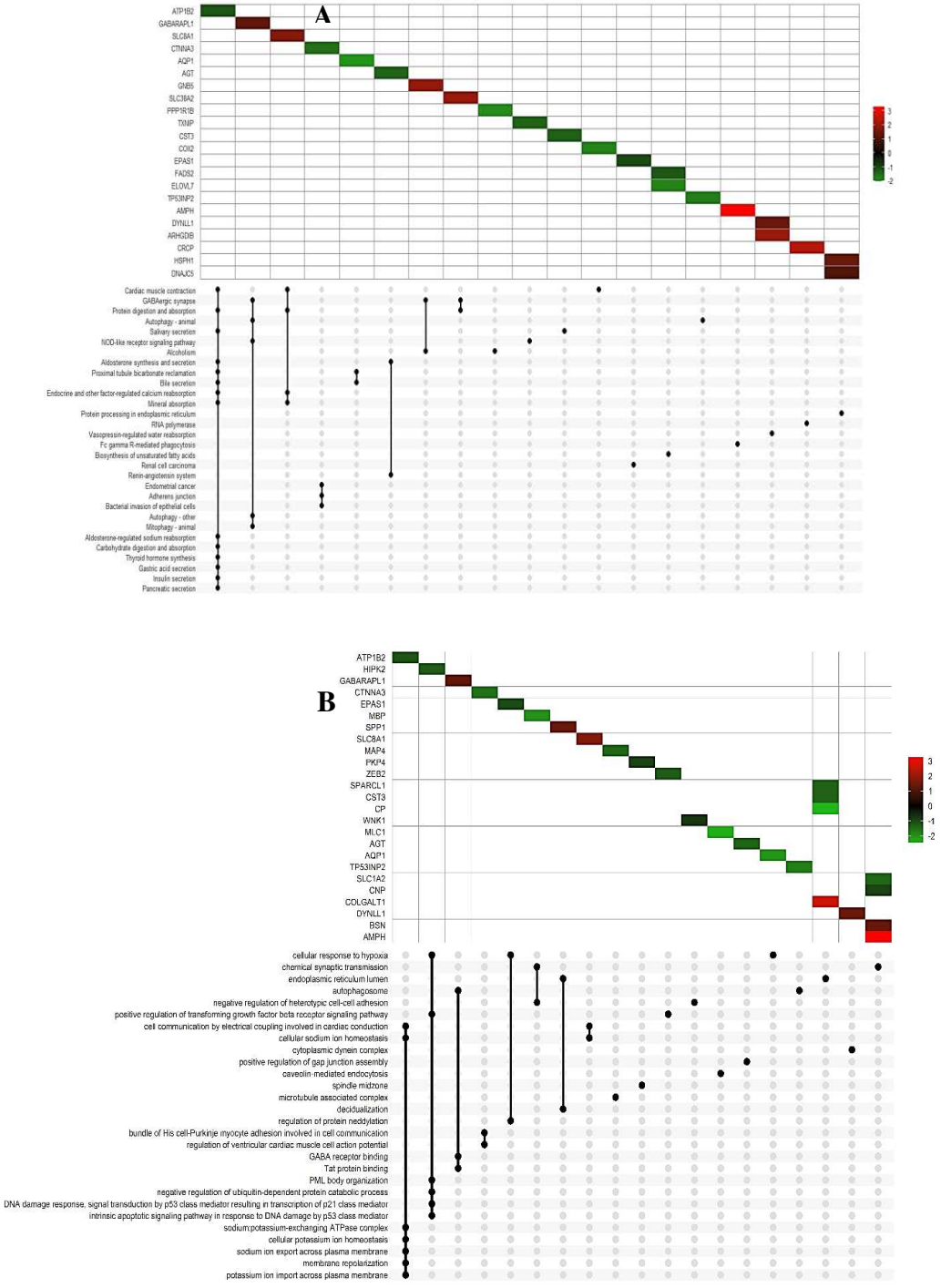

Figure 2. KEGG and GO enrichment analysis of differentially expressed genes. (A) UpSet Plot shown the intersections of significant genes and top 30 enriched KEGG pathway and (B) GO terms, with the Log2FoldChange scale.

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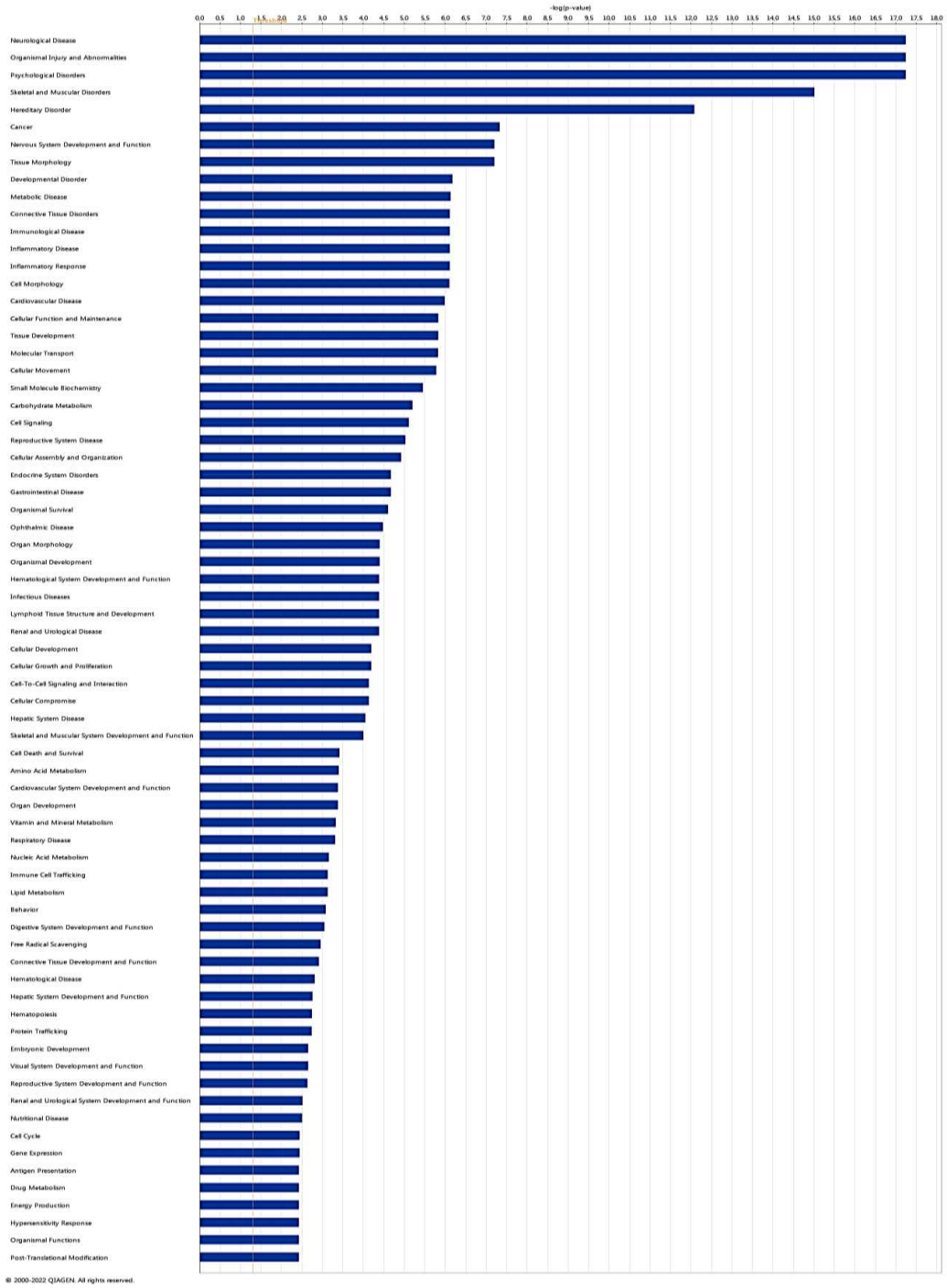

Figure 3. Ingenuity Pathway Analysis (IPA). Diseases and Function analysis of differentially expressed genes in IPA software with Log2 (p-value) scale.

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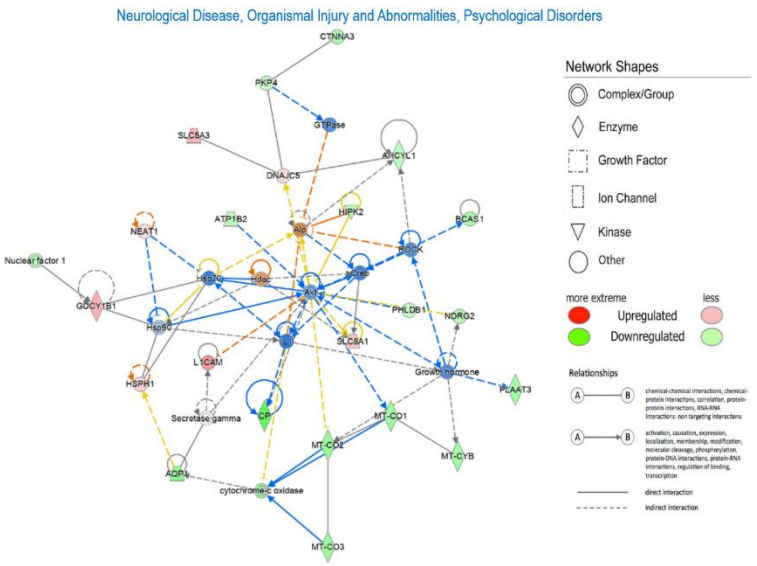

Figure 4. Network Ingenuity Pathway Analysis (IPA). Neurological Disease, Organismal Injury and Abnormalities, Psychological Disorders. Red indicates the upregulated transcripts, whereas green the downregulated transcripts.

Table 3. Networks obtained by IPA Core Analysis.

Molecules in network	Score	Focus molecules	Diseases and functions
AHCYL1, Akt, Alp, AQP1, ATP1B2, BCAS1, CP, Creb, CTNNA3, cytochrome-c oxidase, DNAJCS, Growth hormone, GTPase, GUCY1B1, Hdac, HIPK2, Hsp70, Hsp90, HSPH1, IL1, L1CAM, MT-C01, MTC02, MT-C03, MT-CYB, NDRG2, NEAT1, Nuclear factor M1, PHLDB1, PKP4, PLAAT3, ROCK, Secretase gamma, SLCSA3, SLC8A1	49	22	Neurological Disease, Organismal Injury and Abnormalities, Psychological Disorders
14-3-3, 20s proteasome, 26s Pro teasome, ALAD, BSN, Calmodulin, calpain, CG, CNP, COLGALT1, Collagen Alpha1, Collagen type I (complex), Collagen type IV, EPAS1, ERK1/2, FAR1, Focal adhesion kinase, GFAP, HEPACAM, IN A, insulin, MAP4, MBP, MLC1, PDGF BB, Pka, PP2A, SEPTIN4, SLC1A2, SRGN, TAGLN, Tgf beta, transglutaminase, VSNL1, WNK1	38	18	Cellular Function and Maintenance, Nervous System Development and Function, Tissue Development
AGT, AMPH, Ap1, ARHGDIB, Calcineurin protein(s), CD3, collagen type I (family), cytokine, ELOVL7, FYB1, GNBS, Gsk3, IKK (complex), IL12 (complex), integrin, integrin alpha L beta 2, Jnk, LDL, MAP4K4, Mek, MTURN, NFAT (complex), Nfat (family), NFIX, NFkB (complex), Nrlh, P38 MAPK, PAD12, Pkc(s), PPP1R1B, Rac, SPARCL1, SPP1, TCR, voltage-gated calcium channel Act in, AMPK, Ck2, C LEC9A, CST3, DYNLL1,	25	13	Cardiovascular Disease, Cell-To-Cell Signaling and Interaction, Organismal Injury and Abnormalities
Actin, AMPK, Ck2, C LEC9A, CST3, DYNLL1, ERK, F Actin, FADS2, FAM 107 A, GABARAPL1, G PR34 , hemoglobin, Histone h3, Histone h4, IgG, IL1ORA, IL12 (family), immunoglobulin, interferon alpha, Mapk, MHC Class II (complex), Notch, P13K (complex), RNA polymerase 11 ,	20	11	Cell-To-Cell Signaling and Interaction, Infectious Diseases, Organismal

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SHTN1, Siglech, SRC (family), trypsin, tubulin, TXNIP, Ubiquitin, Vegf, YWHAG, ZEB2			Injury and Abnormalities
ACOD1, CARD16, CASP8, Cd24a, COL2A1, cytokine receptor, Dglucose, DAAM2, FAT3, GBPS, HCAR2, HEPACAM2, IFNG, ligp1, IL10RB, IL17RE, IL18BP, IL2RA, IRAGl , LGALS1, LTC4S, MLKL, NAA30, NLRCS, PARVG, PLAAT3, PRDM11, QDPR, REL, RHOBTB3, SCARA3, Tlr11, TNFRSF10B, TP531NP2, ZBP1	18	10	Gastrointestinal Disease, Inflammatory Response, Organismal Injury and Abnormalities
CFB, CHADL, CNTLN, CSNK1A1, EP300, ETNPPL, FAM110D, FAM83G, FRMD4A, FRY, HDAC4, HDAC5, IKZF2, IL15RA, importin alpha, MECOM, miR-129-Sp (and other miRNAs w/seed UUUUUGC), MOBP, NRBP2, PAIP2B, PAQR6, PDCD1LG2, PPDPF, PRMT1, RCSD1, SCN8A, SMARCB 1, SNX22, SNX24, SOX2, SOX9, TNP01, TSC22D4, ZDBF2, ZNF 618	18	10	Carbohydrate Metabolism, Cell Cycle, Cellular Assembly and Organization
ACBD7, betaestradiol, CA2, CALCRL, Clathrin, CRCP, DEFB116, DNPH1, DOCK3, ERBB, FMOS, HS6ST3, HTR4, INP P5F, L-histidine, L1CAM, Ly6a (includes others), MAL, NOS1, OGDHL, OGN, Pplc, PROTEASE, PTEN, PTPRT, PYGL, SEMA3A, sGC, SLC38A2, SLC02B1, SRC, sulfotransferase, SULT1C2, TBC1D24, Wap	10	6	Cellular Development, Connective Tissue Development and Function, Skeletal and Muscular System Development and Function

Among the genes of interest, GABARAPL1 was found to be overexpressed, as indicated by both KEGG and GO analyses, involving a distinct set of pathways (5 and 3, respectively): GABAergic synapse, autophagy – animal, NOD-like receptor signaling pathway, autophagy – other, and mitophagy – animal (according to KEGG), as well as autophagosome, GABA receptor binding, and Tat protein binding (according to GO). Autophagy, a highly conserved cellular degradation process regulated by specific autophagy-related (Atg) factors, entails the formation of double-membrane autophagosomes that engulf cytoplasmic components for degradation. In mammals, this process is complex due to the presence of six Atg8 homologues, categorized into the GABA type A receptor-associated protein (GABARAP) and microtubule-associated protein 1 light chain 3 (MAP1LC3) subfamilies [39]. GABARAPL1/GEC1, a member of the GABARAP subfamily, exhibits the highest mRNA expression among Atg8 homologues in the CNS. Notably, GABARAPL1 brain expression is observable as early as embryonic day 11, increasing progressively to peak in adulthood. Significantly, GABARAPL1 expression in the adult brain is particularly intense in neurons involved in motor and neuroendocrine functions, notably in the SNc [40]. Dysregulation of Atg8 homologues has been observed in other synucleinopathies, such as Lewy-body dementia and multiple system atrophy [41]. In PD, alterations in autophagic mechanisms are evident, as demonstrated by transcript levels of several autophagy genes in blood cells. A recent study found overexpression of autophagy-related genes, including MAP1LC3B, GABARAP, GABARAPL1, GABARAPL2, and sequestosome 1 (P62/SQSTM1) in PD patients, with potential implications for predicting markers and therapeutic responses [42].

Similarly, the SLC38A2 gene was overexpressed and associated with two relevant pathways according to KEGG: GABAergic synapse and protein digestion and absorption. This finding strengthens the connection between GABA overexpression, PD pathology, and neural degeneration. In a recent study on a rotenone-induced PD rat model [43], nardosinone, a biochemical compound enhancing NGF-mediated neurite outgrowth and synaptogenesis, demonstrated anti-PD efficacy.

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Transcriptome and proteome analysis suggested that the anti-PD target of nardosinone is the SLC38A2 gene, potentially involving the GABAergic synaptic pathway. This underscores the SLC38A2 gene as a potential target for PD treatment and the modulatory effects of this gene as anti-PD agents through the GABA system [43].

Diseases and Function analysis in the IPA software highlighted that DEGs correlated with Neurological Disease (Figure 3, Figure 4A, Figure 4B). Network IPA Analysis (Figure 4B) revealed downregulation of many mitochondrial genes in the SN of PD subjects, including MT-CO1, MT-CO2, MT-CO3, MT-CYB, and Cytochrome-oxidase. This further supports mitochondrial dysfunction in PD, where mitochondria, involved in crucial functions, primarily energy generation, are essential for nearly all cellular activities. Alterations in mitochondrial functioning lead to insufficient energy production, particularly affecting the CNS [45]. Evidence indicates that mitochondrial respiratory chain dysfunction plays a primary role in various neurodegenerative diseases, including PD [45,46]. Impaired elements of the respiratory chain, such as defects in complex I, have been associated with PD and frontal cortex dysfunction [47–50]. Damage to the electron transport chain increases oxidative stress and neuronal dysfunction, potentially contributing to the onset and progression of PD. Progressive mitochondrial damage results in the accumulation of non-functional mitochondria, further contributing to neuronal degeneration [53].

Looking at the downregulated genes in the SN of PD patients, a group of genes involved in maintaining the structure and function of glial cells was noted. These genes include MBP (myelin basic protein), CPN (myelin protein cyclic nucleotide phosphodiesterase), CTNNA3 (alpha-T-catenin), AQP1 (aquaporin 1), and GFAP (glial fibrillary acidic protein). Among the upregulated genes, SLC8A1 (solute carrier family 8 member A1) is involved in linking trans-plasmalemmal gradients of sodium and calcium ions to the membrane potential of astrocytes. This outcome suggests the emerging important role of glial cells in neurodegeneration and PD pathogenesis [59].

A limitation of this study is the small number of PD and control subjects recruited; however, the ability to sample human brains and extract mRNA sequences directly from the SN of both patients and controls is a significant strength of this report.

3. Materials and Methods

3.1. Human post-mortem midbrain samples

Human midbrain samples were generously provided by the Parkinson's UK Brain Bank (Imperial College London, London, UK). A total of 8 PD and 6 CTRL samples were acquired, and the specimens were histologically sectioned at the midbrain, encompassing the human SN in all slides. Each section had a thickness of 4 μm. Supplementary Table 4 provides a summary of the clinical features of the included subjects. The study adhered to the principles of the Declaration of Helsinki of 1964 and its subsequent amendments. The Ethics Committee of the Oasi Research Institute—IRCCS of Troina (Italy) granted approval for the protocol on April 5th, 2022 (approval code: 2022/04/05/CE-IRCCS-OASI/52).

3.2. RNA isolation from human midbrain samples

RNA was extracted from 4 μm FFPE slide-mounted sections using the RecoverAll Total Nucleic Acid Isolation Protocol (ThermoFisher Scientific), following the manufacturer's instructions. Subsequently, the RNA was stored at -80°C until further processing.

3.3. RNA Sequencing and Functional Analysis

RNA sequencing and subsequent data analysis were conducted by Genomix4Life Srl (Baronissi, Italy). The quality and quantity of RNA were assessed using a Qubit fluorometer (Thermo Fisher Scientific) and a TapeStation 4200 (Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA, USA), respectively.

Indexed libraries were prepared from 50 ng of purified RNA each, employing the Illumina Stranded Total RNA with Ribo-Zero Plus Kit (Illumina), following the manufacturer's guidelines.

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Library quantification was performed using the TapeStation 4200 (Agilent Technologies) and Qubit (Thermo Fisher). Subsequently, the indexed libraries were pooled in equimolar amounts, resulting in a final concentration of 2 nM. The Illumina NovaSeq 6000 System was utilized to sequence the pooled samples in a 2 × 75 paired-end format.

Quality control analysis was executed on the raw fastq files generated earlier using the FastQC tool [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/]. Trim-ming of short reads (<25 bp) and removal of adapter sequences were carried out with cutadapt (v.2.8) [60]. The trimmed fastq files were mapped to the reference genome (GenCode (HG38-Release 37 (GRCh38.p13)) [https://www.gencodegenes.org/human/]) using STAR (version 2.7.3a) [61] with standard parameters. Gene quantification per sample was accomplished using featureCount (version 2.0) [https://bioweb.pasteur.fr/docs/modules/subread/1.4.6-p3/SubreadUsersGuide], and a custom R script was employed for data normalization and differential expression analysis using the Bioconductor DESeq2 [62] package. The threshold for considering genes as differentially expressed was set at Fold-Change ≥ 1.50 or ≤ -1.50 (|FC| ≥ 1.50) with adjusted p-values ≤ 0.05 (padj). Volcano plots and heatmaps were generated using the EnhancedVolcano (10.18129/B9.bioc.EnhancedVolcano) and ComplexHeatmap [63] packages in R.

For functional analysis, KEGG pathway and GO database analyses were conducted using the R package pathfinder [https://doi.org/10.3389/fgene.2019.00858]. Additionally, to gain a deeper understanding of the complex transcriptomics data, Ingenuity Pathway Analysis (IPA) [64] was performed, specifically for investigating diseases and function analysis.

The raw data (.fastq files) and the normalized count of identified mRNAs are available on ArrayExpress (E-MTAB-13295).

4. Conclusions

Despite the limited sample size, the comprehensive analysis conducted on the transcriptome of these samples, along with KEGG and GO enrichment analyses, revealed statistically significant findings associated with the following signaling pathways: cardiac muscle contraction, GABAergic synapse, autophagy, Fc gamma R-mediated phagocytosis signaling pathway, cellular response to chemical synaptic transmission to hypoxia, autophagosome assembly, GABA receptor binding, and cellular communication via electrical coupling involved in cardiac conduction.

Upon examining the genes that were either upregulated or downregulated, these results contribute to a translational perspective and unveil novel pathophysiological insights into PD. This includes implications in cell communication via electrical coupling, specific mitochondrial activity, and neuronal-glia interaction. These findings may pave the way for enhanced diagnostic precision and the development of novel targeted therapies.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Table 1: KEGG pathway database and enrichment analysis; Supplementary Table 2: GO pathway database and enrichment analysis; Supplementary Table 3: KEGG pathway database and enrichment analysis; Supplementary Table 3: Diseases and Biological Functions; Supplementary Table 4: main clinical features on the subjects included in the study.

Author Contributions: Concept and Design, M.S., G.C., and R.F.; performed Next generation sequencing analysis, M.R., G.M., and G.M.V.; acquisition of data or analysis, M.R., G.M., and G.M.V. and M.S.; writing original draft preparation, M.S., G.L., M.R., G.M., F.A.S., M.G.S., G.C., S.M., and R.F.; Final approval, M.S., G.L., M.R., and R.F. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki of 1964 and its later amendments and the Ethics Committee of the Oasi Research Institute-IRCCS of Troina (Italy) approved the protocol on April 5th, 2022 (approval code: 2022/04/05/CE-IRCCS-OASI/52).

Informed Consent Statement: The Centre for Blast Injury Studies fully complies with The Human Tissue Act 2004 (which replaced the Human Tissue Act 1961, the Anatomy Act 1984 and the Human Organ Transplants Act 1989), governed by the Human Tissue Authority, which outlines the use of human tissue for scientific purposes in the UK. Although the Human Tissue Act does not cover the use of tissues that are sourced outside the UK,

the Centre also complies with its standards in its dealings with human tissue from overseas. Imperial College London also holds an institutional license from the Human Tissue Authority to collect, store and use human tissue. As provided for in the Human Tissue Act, and depending on the nature of the research, organizations other than the Human Tissue Authority regulate the actual research on human tissue and in the case of this study this has been via Ethics Committee of the Oasi Research Institute-IRCCS of Troina (Italy). .

Data Availability Statement: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (E-MTAB-13295).

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