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Network analysis identifies disease-specific pathways for Parkinson's disease.

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Abstract

Neurodegenerative diseases are characterized by the progressive loss of specific neurons in selected regions of the Central Nervous System. The main clinical manifestation (movement disorders, cognitive impairment, and/or psychiatric disturbances) depends on the neuron population being primarily affected. Parkinson's disease is a common movement disorder, whose aetiology remains mostly unknown. Progressive loss of dopaminergic neurons in the substantia nigra causes an impairment of the motor control. Some of the pathogenetic mechanisms causing the progressive deterioration of these neurons are not specific for Parkinson's Disease but are shared by other neurodegenerative diseases, like Alzheimer Disease and Amyotrophic Lateral Sclerosis.

Here, we performed a meta-analysis of the literature of all the quantitative proteomic investigations of neuronal alterations in different models of Parkinson's Disease, Alzheimer Disease and Amyotrophic Lateral Sclerosis, to distinguish between general and Parkinson's Disease specific pattern of neurodegeneration. Then, we merged proteomics data with genetics information from the DisGeNET database. The comparison of genes and proteins information allowed us to identify 25 proteins involved uniquely in Parkinson's Disease and we verified the alteration of one of them, i.e., Transaldolase 1 (TALDO1), in the substantia nigra of 5 patients. By using open-source bioinformatics tools, we identified the biological processes specifically affected in Parkinson's Disease, i.e., proteolysis, mitochondrion organization, mitophagy. Eventually, we highlighted four cellular component complexes mostly involved in the pathogenesis: the proteasome complex, the protein phosphatase 2A, the chaperonins CCT complex and the complex III of the respiratory chain.

Keywords: network analysis, pathway analysis, meta-analysis, Parkinson's Disease, Alzheimer Disease, Amyotrophic Lateral Sclerosis

Abbreviations

2-DE - Two-dimensional electrophoresis

AD - Alzheimer Disease

ALS - Amyotrophic Lateral Sclerosis

BCA- Bicinchoninic acid

CCT- Chaperonin containing TCP-1

CNS - Central nervous system

CTRL-Control subjects

DAG -Directed acyclic graph

DTT-1,4-Dithiothreitol

EDTA- Ethylenediaminetetraacetic acid

ER - Endoplasmic reticulum

ETC- Electron transport chain

FTD- Frontotemporal dementia

GO - Gene ontology

GSEA- Gene Set Enrichment Analysis

LBs - Lewy bodies

MPTP-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS - Mass spectrometry

NBB - Netherlands Brain Bank

ORA - Over-representation analysis

PD - Parkinson's disease

PIC- Protease inhibitor cocktails

PMSF- Phenylmethylsulfonyl fluoride

PPI - Protein-protein interaction

PVDF- Polyvinylidene difluoride

ROS- Reactive oxygen species

SDS- Sodium dodecyl sulfate

SN - Substantia nigra

TBST- Tris-buffered saline tween

Introduction

Neurodegenerative disorders are characterized by the progressive deterioration of neuronal systems in the central nervous system (CNS). The loss of neurons results in movement disorders, cognitive impairment, and/or psychiatric disturbances. The pathological defects that underlie neurodegenerative disorders are fairly well defined and usually involve the progressive death of specific neurons in selected regions of the CNS. Despite of their distinct aetiologies, neurodegenerative disorders probably share pathogenetic mechanisms that involve oxidative stress, unfolded protein response and mitochondrial impairment [1]. Although the main clinical manifestations of each disorder depend on the neuron population being primarily affected, some individuals show signs and symptoms indicating the extension of the pathology to other systems [2-4].

Parkinson's Disease (PD) is a multifactorial neurodegenerative disorder, whose aetiology remains largely unknown. Early in the course of the disease, the symptoms are movement-related, including tremor, rigidity, slowness of movement and difficulty with walking and gait [5,6]. Progressive impairment of motor control is caused by the specific loss of dopaminergic neurons in the substantia nigra (SN) and the formation of intra-neuronal proteinaceous inclusions, called Lewy bodies (LBs) [7,8]. The progressive deterioration of vulnerable SN dopaminergic neurons may arise from cellular disturbances caused by misfolding and aggregation of the synaptic protein alpha synuclein [9], the disruption of the autophagy-lysosome system [10], mitochondrial dysfunction [11], endoplasmic reticulum (ER) stress [12], and dysregulation of calcium homeostasis [13]. These pathways are not specific for PD but they are shared by Alzheimer Disease (AD) [14] and Amyotrophic Lateral Sclerosis (ALS) [2] and there is recent evidence supporting the view that ALS and PD share common pathogenetic mechanisms. Indeed, the frequency of extrapyramidal symptoms in ALS patients is significantly higher than in the general population [15,16].

Current research on the molecular bases of neurodegenerative disorders using omics technologies (e.g., genomics, proteomics, and metabolomics) have enabled high-throughput

monitoring of a variety of molecular and organismal processes. These techniques have been generally applied to identify biological variants, to characterize the involved complex biochemical systems and to study pathophysiological processes. While many omics platforms target comprehensive analysis of genes (genomics), mRNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) [17], challenges remain for omics-domain data integration [18]. Changes in mRNA levels do not always correlate with similar changes in protein levels or activity, therefore studies evaluating protein expression changes in neurodegenerative models are crucial [19]. The most common methods of proteome analysis are the comparative two-dimensional electrophoresis (2-DE), followed by mass spectrometry (MS), and shotgun proteomics, based on a gel-free approach. 2-DE shows a better workbench for the characterization of post-translational modifications. However, shotgun proteomics provides a better sensitivity and resolution [20].

In the present study we analyzed the biological processes that result to be altered in PD, making a comparison with two other neurodegenerative disorders; AD, as the most studied neurodegenerative condition, and ALS, as a movement disorder affecting the motor neurons, instead of a midbrain neuron population as in PD. To this end, we performed a meta-analysis of the literature encompassing all the proteomic investigations published so far concerning neuronal alterations in PD, ALS and AD. Unlike the meta-analysis of clinical studies, we collected all data on proteins that have been reported to be quantitatively altered in either diseases. We then combined these data with disease-linked genes taken from curated disease-gene databases (DisGeNET) [21] in order to unveil disease-specific biological pathways. This allowed us to obtain an enriched dataset for the following systems biology analysis [19].

Materials and methods

Input lists generation

A meta-analysis of the literature was obtained by gathering all proteomic investigations of neuronal alterations in PD, ALS and AD. The search was conducted in PubMed database, using the following search terms: “proteom*[Title/Abstract]) AND Parkinson* [Title/Abstract]”. All English language studies found in the database and published within the end of 2015 were considered. Only full text original articles focused on the development of the disease in the CNS were included. Therefore, all papers on peripheral biomarkers and on the drug influence on the pathogenesis were manually filtered out. All protein IDs were converted to the corresponding human Uniprot ID. Genetic information taken from the curated database DisGeNET (<http://disgenet.org>, v4 released April 15th 2016) were also collected. The following search terms were used in DisGeNET: PD (umls:C0030567), ALS (umls:C0002736) and AD (umls:C0002395).

Bioinformatics analysis

By merging the protein input lists for the three diseases, proteins were divided between disease-specific (only-PD_Proteins, only-ALS_Proteins and only-AD_proteins) and in common among different disease states. In order to identify genes and proteins specific for PD (PD_Proteins-Genes), the tool InteractiVenn was used (<http://www.interactivenn.net/>) [22].

The over-representation analysis (ORA) was carried out using the GO Consortium (<http://geneontology.org/>) and Reactome (<http://www.reactome.org/>) databases. Only significant pathways (p -value < 0.05) were taken in account. In order to compare the results obtained after the analysis of the input lists, we used GOView (<http://www.webgestalt.org/GOView/>). GOView is a web-based application which allows both visualization and comparison of multiple GO term lists in a directed acyclic graph (DAG) able to unveil relationships among terms.

Networks were built using the web portal BioProfiling (<http://www.bioprofiling.de>). This tool includes almost all the available information concerning signalling and metabolic pathways

(databases: Reactome and KEGG) and physical protein-protein interaction (PPI) (database: IntAct) [23]. Accordingly, the protein input list was analysed with the PPI spider [24] tool. The p-value provided, computed by Monte Carlo simulation, refers to the probability to get a model of the same quality starting from a random gene list of the same size [23]. The significant D1 networks (all nodes belong to the input list, $p < 0.01$) were considered to further interpret and discuss proteomics results. The networks were exported as .xgmml files and edited using Cytoscape 3.2.0 (<http://www.cytoscape.org/>) [25].

To perform network topology analysis of the enriched networks, we used the NetworkAnalyzer Cytoscape application. NetworkAnalyzer performs analysis of biological networks providing network topology parameters including the diameter of a network, the average number of neighbours and the number of connected pairs of nodes. Distributions of more complex network parameters such as node degrees, average clustering coefficients, topological coefficients and shortest path lengths were also computed [26]. Again, the GO Consortium was used to perform ORA (GO terms).

Western blot analysis of SN samples

Mesencephalic tissues from five sporadic PD patients and five controls (defined by the Brain Bank as non-demented, since they did not show any sign of dementia at the time of death) (Supplementary Table 1) were obtained from the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. All material has been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB.

The tissue specimens (approximately 12 mg) were put in a Potter homogenizer together with 500 μ l of tissue lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton, 2 mM ethylenediaminetetraacetic acid (EDTA) 1 mM 1,4-dithiothreitol (DTT), 1x phosphatase inhibitors (Roche), 1x phenylmethylsulfonyl fluoride (PMSF), 1x protease inhibitor cocktails (PIC) and then

manually lysed. After this procedure, the samples were incubated on ice for 30 minutes and then sonicated (0.5 cycles, 15 pulses, twice). Then, the lysates were centrifuged at 18000×g for 15 minutes at 4°C.

Total protein concentration was quantified using the bicinchoninic acid (BCA) method (Pierce).

Equal amounts of proteins were resolved in 10% sodium dodecyl sulfate (SDS, TGX Stain-Free FastCast Acrylamide Kit, 10%, BioRad) and the fluorescent stain activated by UV light, following manufacturer's instructions. Then, proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 1.0 mA/cm² for 2 hours (TE77pwr; Hoefer) and the fluorescent signal acquired (GelDoc-It Imaging System; UVP). Membranes were saturated for 2 hours at room temperature in tris-buffered saline tween (TBST), integrated with 5% of skimmed milk powder and then incubated with anti-TALDO1 (Abcam ab67467, 1:500) and β-actin (GeneTex GTX23280, 1:8000) primary antibody overnight at 4°C, in 5% milk-TBST. Incubation with anti-mouse (Millipore 12-349, 1:1500) peroxidase-conjugated secondary antibody in 5% milk-TBST was then performed. Enhanced chemiluminescence substrate (Millipore Corporation) was used in order to visualize the peroxidase signals: images (16 bit grayscale) were finally acquired using the G:BOXChemi XT4 (Syngene) system and analyzed using the ImageJ software [27] (<https://imagej.nih.gov/ij/>), normalizing each TALDO1 signal for the β-actin signal.

Results

Assembly of gene and protein lists

In order to gather valuable information for the assembly of the input lists, a meta-analysis of the literature was performed, including all the proteomic investigations of AD, PD and ALS disease models. Full-texts of the selected articles were used to extract information about proteins altered by the three diseases and to generate the input lists (Table 1). We identified 762, 420 and 139 papers in PubMed about proteomic investigations in AD, PD and ALS, respectively. The studies retained (healthy/disease comparison and quantitative proteomic studies) were 40 for AD, 46 for PD and only 9 for ALS (Supplementary Table 2). We generated three input lists: AD_Proteins (928 proteins), PD_Proteins (1155 proteins) and ALS_Proteins (387 proteins) (Table 1).

Table 1. Principal steps of the meta-analysis procedure and number of IDs in each input list.

	Pubmed hits	Healthy/disease comparisons and quantitative proteomic studies	_Proteins	_Genes
AD	762	40	928	1663
PD	420	46	1155	766
ALS	139	9	387	799

We analyzed the three lists in order to identify proteins that were not unique to a specific disease (Fig. 1). On the other hand, we observed that 675 proteins resulted to be altered in PD only (only-PD_Proteins input list). As far as the intersections are concerned, PD and ALS shared 69 proteins, while AD and ALS showed only 29 proteins in common.

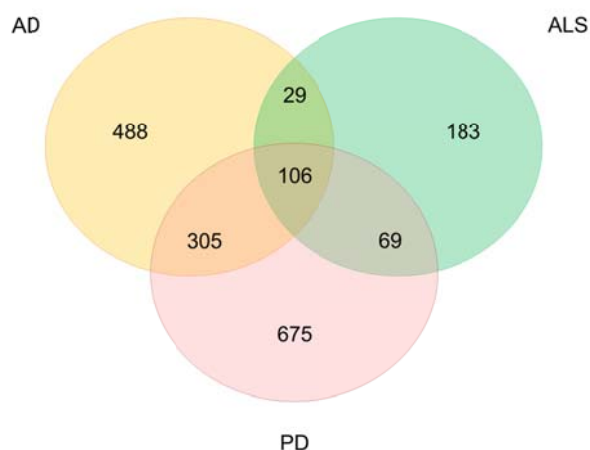


Fig. 1 : Venn diagram showing the number of disease-specific proteins and proteins altered by more than one disease

We then included genetic information extracted from the curated database DisGeNET and we obtained three additional input lists called AD_Genes, PD_Genes and ALS_Genes, respectively (Table 1).

In order to identify both proteins and genes exclusively associated with PD, we used the InteractiVenn tool (Fig. 2). As a result, we identified 25 IDs that were present in both PD_Proteins input list and PD_Genes input list and not present in any other protein or gene list. The 25 IDs were: Aromatic-L-amino-acid decarboxylase (DDC), GTP cyclohydrolase 1 (GCH1), Disks large homolog 2 (DLG2), Fructose-1,6-bisphosphatase 1 (FBP1), 60S ribosomal protein L6 (RPL6), 60S ribosomal protein L23a (RPL23A), 40S ribosomal protein S8 (RPS8), 60S ribosomal protein L14 (RPL14), Transaldolase 1 (TALDO1), Ribosyldihyronicotinamide dehydrogenase [quinone] (NQO2), Glutamate decarboxylase 1 (GAD1), OX-2 membrane glycoprotein (CD200), Wolframin (WFS1), Pyridoxal kinase (PDXK), AP-1 complex subunit mu-1 (AP1M1), Beta-2-microglobulin (B2M), Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), Sideroflexin-2 (SFXN2), Fibrillin-1 (FBN1), Glutamate dehydrogenase 2, mitochondrial (GLUD2), Heat shock 70 kDa protein 1-like (HSPA1L), Retinal dehydrogenase 1 (ALDH1A1), Mesencephalic astrocyte-

derived neurotrophic factor (MANF), Small nuclear ribonucleoprotein F (SNRPF), Ubiquitin-conjugating enzyme E2 S (UBE2S) (PD_Proteins-Genes input list) (Supplementary Table 3).

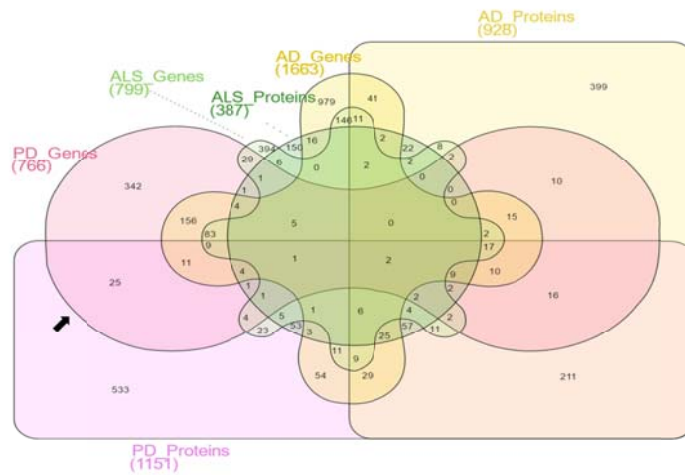


Fig. 2: Venn diagram showing disease-specific genes and proteins and those shared by different diseases. The arrow shows the overlap between the PD_Proteins list and the PD_Genes list

Verification of Transaldolase 1 as a PD protein

As a proof of principle, we selected one protein in the list of the 25 PD unique IDs to verify its up-regulation in PD affected tissues, as suggested by the bioinformatics analysis. We checked TALDO1 abundance in SN samples obtained from five sporadic PD patients and five matched control subjects. As shown in Fig. 3, we verified TALDO1 increase in the affected tissue of sporadic PD patients.

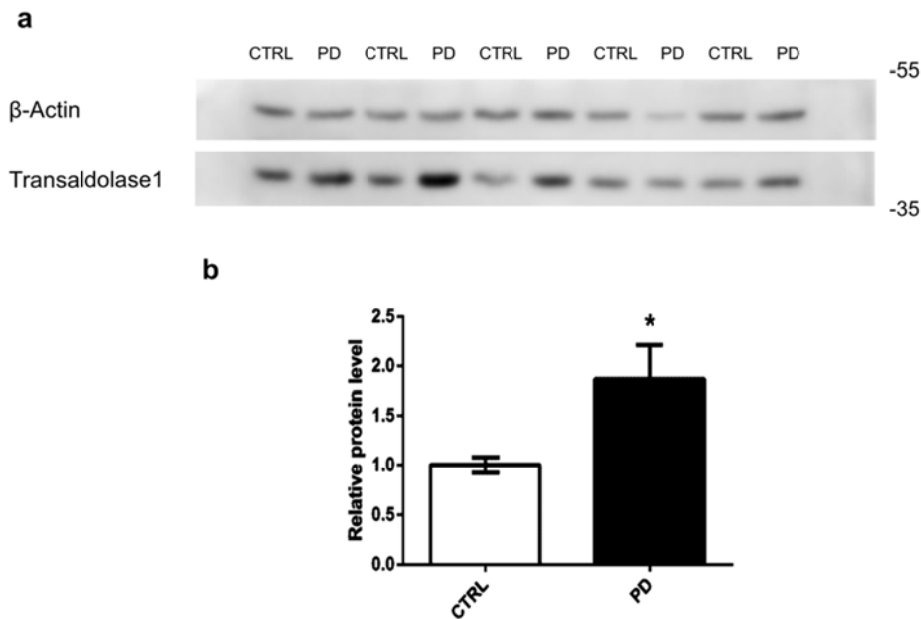


Fig. 3: Western blot analysis of 10 SN specimens, coming from 5 PD patients and 5 control subjects (CTRL). (a) A representative Western blot, showing Transaldolase 1 and β -Actin, used as a loading control. (b) Bars report the relative change of TALDO 1 signals (normalized to the related β -actin signal) in PD patients with respect to the control subjects, arbitrarily set to 1. Values are the mean \pm SEM of three technical replicates (*, $p < 0.05$)

Over-representation of biochemical pathways and gene ontology categories

In order to understand which cellular pathways are mainly affected by the three disease conditions examined, an ORA was performed on the six input lists (the three “_Proteins” input lists and the three “_Genes” input lists), using the gene ontology (GO) enrichment tool of the GO consortium. Given the huge amount of results obtained, we used GOView to select and sum up the most relevant information (Table 2 and Table 3).

Table 2. Summary table of the most significant results of the GO Biological Processes analysis of “_Proteins” input lists, after GoView filtering.

AD_Proteins	PD_Proteins	ALS_Proteins
<p style="text-align: center;">Apoptotic process Electron transport chain Axon guidance Intracellular protein transport Protein targeting Signaling pathway (Fc receptor, NIK/NF-kappaB)</p>		
	<p style="text-align: center;">Cell cycle phase Establishment of protein localization to endoplasmic reticulum DNA damage response Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay</p>	
<p style="text-align: center;">Negative regulation of neuron apoptotic process Establishment of synaptic vesicle localization Mitochondrial membrane organization Mitochondrial transport Signaling pathway (ERBB, Fc epsilon, fibroblast growth factor receptor, insulin receptor, epidermal growth factor receptor)</p>		
G2/M transition of mitotic cell cycle		G2/M transition of mitotic cell cycle
Cellular potassium and sodium ion homeostasis Microtubule cytoskeleton organization	Proteolysis involved in cellular protein catabolic process Mitochondrion localization Mitophagy Signaling pathway (ephrin receptor, tumor necrosis factor, canonical Wnt)	rRNA metabolic process ncRNA metabolic process Nuclear transport RNA transport

Table 3. Summary table of the most significant results of the GO Biological Processes analysis of “_Genes” input lists, after GoView filtering.

AD_Genes	PD_Genes	ALS_Genes
Regulation of cellular catabolic process Regulation of peptidyl-serine phosphorylation Regulation of protein transport Regulation of apoptotic signaling pathway		
Regulation of myelination Positive regulation of nuclear division		
Cellular response to xenobiotic stimulus Regulation of mitochondrial membrane permeability Calcium ion transport Signaling pathway (ERBB, Fc receptor)		
Positive regulation of glial cell differentiation Low-density lipoprotein particle remodeling Cellular response to glucose stimulus Ionotropic glutamate receptor signaling pathway Regulation of phosphatidylinositol 3-kinase signaling		Positive regulation of glial cell differentiation Low-density lipoprotein particle remodeling Cellular response to glucose stimulus Ionotropic glutamate receptor signaling pathway Regulation of phosphatidylinositol 3-kinase signaling
Positive regulation of glycolytic process Icosanoid biosynthetic process Regulation of fatty acid biosynthetic process Positive regulation of cholesterol transport Signaling pathway (Notch, Wnt, Toll-Like Receptors, tumor necrosis factor)	Dopamine biosynthetic and catabolic process Response to oxygen radical Apoptotic mitochondrial changes Cellular iron ion homeostasis	RNA transport Nuclear import mRNA splicing, via spliceosome Peptidyl-cysteine S-nitrosylation

The “Apoptotic process” served as an internal control, since alterations in the apoptotic pathway resulted to be present in all of the three diseases (PD, AD and ALS). Similarly, the ORA analysis unveiled the alteration of protein transport as a common feature of the three diseases (“Protein targeting” and “Regulation of protein transport”). On the other hand, the ORA allowed us to identify disease-specific pathways. Indeed, in PD we recognized a central role for mitochondria both in “_Proteins” (“Mitophagy” and “Mitochondria localization”) and in “_Genes” analyses (“Apoptotic mitochondria change”). Furthermore, alterations in proteasomal function (“Proteolysis involved in cellular protein catabolic process”) and “Response to oxygen radical” were observed. By contrast, cytoskeleton organization, ion homeostasis and lipid biosynthetic process resulted to be altered in AD, while aberrant RNA processing appeared to be mainly involved in ALS pathogenesis (“rRNA metabolic process”, “ncRNA metabolic process”, “mRNA splicing, via spliceosome”).

In order to identify which pathways are over-represented when only PD-specific proteins are considered, an ORA of only-PD_Proteins input list (675 IDs) was performed. To this purpose, we used GO Consortium Biological Process and Reactome as reference databases. Again, we found “Mitophagy” and apoptotic process (“Regulation of apoptosis”), but we also found out “Translation” and “Metabolism of amino acid and derivatives” (Table 4).

Table 4. Summary table of the most significant results of the GO Biological Processes and Reactome analysis of only PD_Proteins input list.

Only-PD_Proteins (675 IDs)	
Go Consortium Biological Process	Reactome
Vesicle-mediated transport Regulation of apoptotic process Regulation of cell motility Protein complex assembly Signaling pathway (MAPK cascade, insulin receptor signaling pathway, epidermal growth factor receptor signaling pathway, vascular endothelial growth factor receptor signaling pathway, neurotrophin TRK receptor signaling pathway, fibroblast growth factor receptor signaling pathway, NIK/NF-kappaB signaling, Wnt signaling pathway, planar cell polarity pathway) Regulation of protein catabolic process Actin cytoskeleton organization Modulation of synaptic transmission Mitochondrion organization Translation Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay SRP-dependent cotranslational protein targeting to membrane	Cellular response to heat stress DNA replication Laminin interactions Nonsense-Mediated Decay (NMD) Mitophagy (PINK1/Parkin mediated Mitophagy) Metabolism of aminoacid and derivates Translation Regulation of apoptosis Signaling pathway (signaling by EGFR, signaling by FGFR, signaling by NGF, signaling by PDGF, signaling by VEGF, MAPK family signaling cascade, signaling by Wnt, signaling by Hedgehog) Membrane trafficking

Strikingly, the latter pathways are overrepresented in the PD_Proteins-Genes analysis, too (Supplementary Table 4).

Physical interactions network

Starting from the protein input lists previously generated, one for each disease considered ("_Proteins"), we decided to perform a network-based analysis, using the IntAct database as a reference set. With this approach, we generated a direct interactor network (D1 network) for each disease (Supplementary Fig. 1) and we then merged them (Supplementary Fig. 2). In this way, we excluded those nodes that were shared by different diseases and we thus identified “only-PD-

nodes”. Later, we used this new list of nodes to create new interaction networks (129 nodes). Fig. 4a shows the D1 model for the “only-PD_nodes”. This network highlights the alteration of proteins involved in the regulation of proteolysis (dark green nodes), a process mainly guided by the proteasome complex. Nodes representing proteins that belong to PD_Proteins-Genes list are highlighted in bold.

Afterwards, NetworkAnalyzer was used to investigate which cellular component complexes are involved in PD. We adjusted the size of nodes according to its degree: the bigger the node, the higher the number of edges (Fig. 4b). At the same time, we performed an ORA using the category Cellular Component by GO Consortium to identify the cellular localisation of the complexes. The results obtained with the two different methods are in keeping and showed that four complexes are included in the network: “Respiratory chain complex”, “Proteasome complex”, “chaperonin containing T complex” (CCT complex), “Protein phosphatase 2a complex” (PPP2A complex) (Fig. 4b).

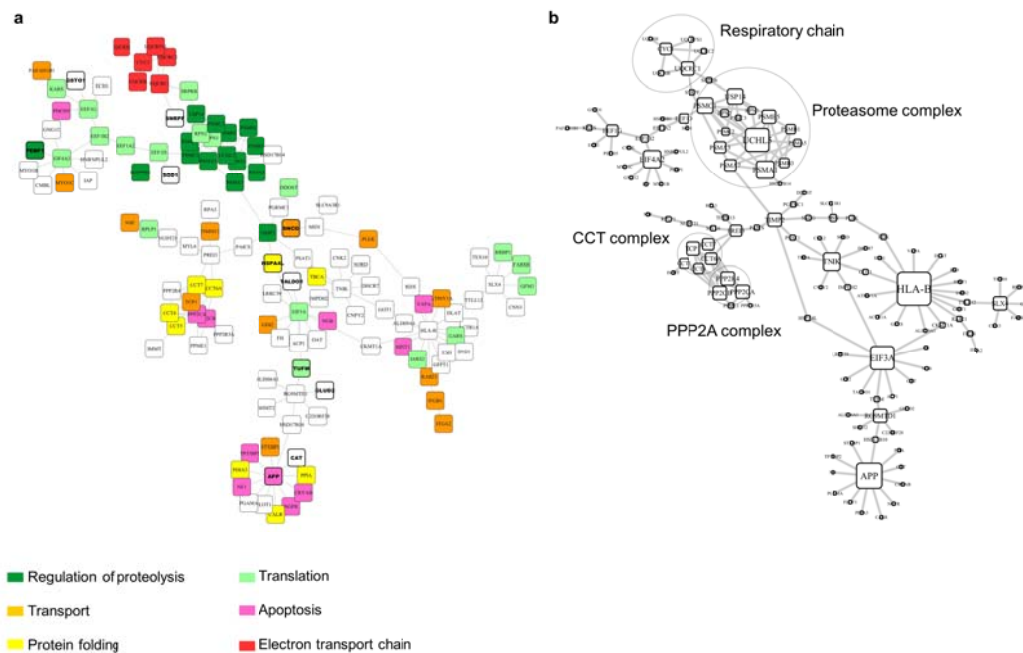


Fig. 4: Significant networks built by PPI spider ($p < 0.01$), using IntAct database as the reference set. (a) D1 model of only-PD_nodes. Nodes in bold are annotated also in the PD_Genes list. Colours indicate gene functional role according to the Gene Ontology classification. (b) D1 model of only-PD_nodes after analysis with NetworkAnalyzer and ORA using GO consortium Cellular Component

Cellular component complex analysis

Eventually, we analysed the complexes separately to assess how they are involved in PD. Quantitative information were retrieved on the original papers: proteins reported as consistently up-regulated in PD are red nodes, while down-regulated ones are green nodes. Yellow nodes represent contradictory results (different studies show opposite variations) (Fig. 5). Alterations in protein level are reported in Supplementary Table 5.

Fig. 5a shows two closely related complexes, the chaperonins CCT complex and the phosphatases PPP2A complex. In the CCT complex, all the chaperonins are down-regulated, with the exception of CCT5 that is down-regulated when SNCA is overexpressed [28] as well as in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated (MPTP-treated) mice [29]. Conversely, it is up-regulated in mouse neuroblastoma cell lines [30] and in PINK1 knockout mice [31]. With reference to the respiratory chain (Fig. 5b), the components are altered in different directions, depending on the disease model considered (three yellow nodes: UQCRH, CYC1 and UQCRC1). In addition, results regarding UQCRC2 and UQCRSF1 are ambiguous (Supplementary Table 5). In the proteasome complex (Fig. 5c), we observed a down-regulation of subunit 19S ATP-dependent components and the up-regulation of subunit catalytic 20S members.

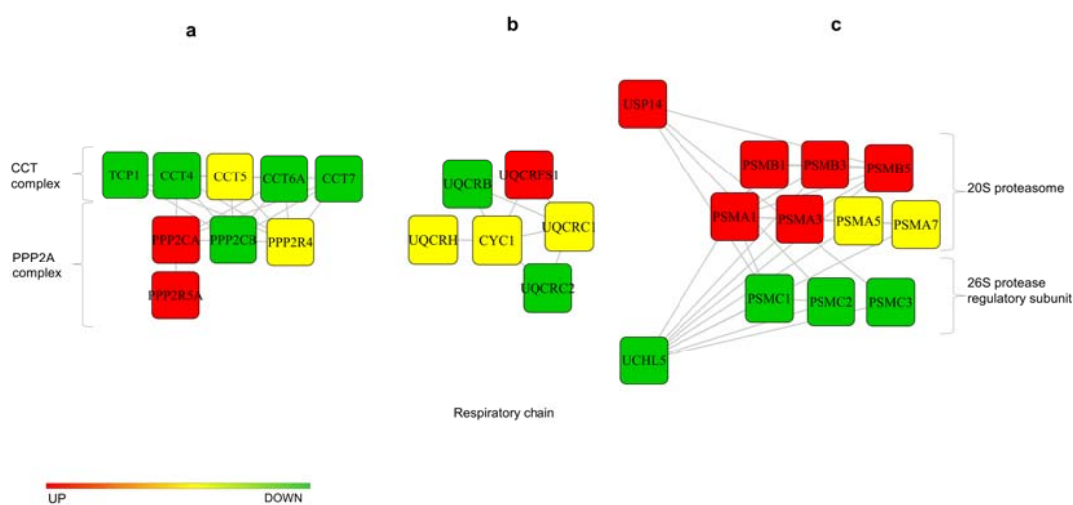


Fig. 5: The nodes are coloured based on their confidence of being up- (red) or down-regulated (green) in the disease state. Yellow nodes are proteins whose level is presented as down-regulated in some papers and up-regulated in others. (a) shows the chaperonins CCT complex and the phosphatases PPP2A complex, (b) shows the complex III of the respiratory chain and (c) represents the proteasome complex

Discussion

Neurodegenerative diseases are multifactorial in nature involving several genetic mutations (in coding or regulatory regions), epigenetic and environmental factors, the greatest risk factor being age [32]. Multifactorial diseases can be investigated using a global approach at the systems level [33]. Proteomics offers a global snapshot at the molecular level of proteins and consequently processes that regulate neuronal death. The integration of this view with genetic information can help to understand general mechanisms of neurodegeneration. To the purpose of finding molecular pathways specifically involved in PD, we used AD as a control, to eliminate pathway generically linked to neurodegeneration. Moreover, we included ALS in the analysis, being another disorder of motor control, although affecting a distinct neuronal population (i.e., motor neurons). The aetiology of AD, PD and ALS remains largely unknown. Therefore, we performed a meta-analysis of the literature and merged the results of only those papers containing proteomics experiments about neuronal modifications in PD, ALS and AD, including papers on human tissues, animal models and cellular models, and excluding works about pharmacological effects of particular molecules or peripheral biomarkers.

The meta-analysis approach presented here may have some limits. First, we decided to include only quantitative proteomics studies. We tried to overcome this issue using data coming from a genetic database (DisGeNET) and taking into account only the “consensus” pathways obtained by the ORA of both proteins and genes lists, in this way overcoming also the issue of false results present in the literature. Another weakness of retrieving data from the literature is the few proteomics information about ALS compared to those of PD and AD. Consequently, the molecular picture obtained for ALS was less complete than the one for the other two diseases. Eventually, the ORA of long lists of proteins/genes generates huge lists of pathways where it is difficult to select those biologically relevant. Having access to primary quantitative data should be a method to filter out some less relevant results, using tools such as the Gene Set Enrichment Analysis (GSEA).

There is an extensive evidence that demonstrates the close relationship between ALS and PD at the clinical [34;35], genetic [15], neuroimaging [36] and pathological level in humans [37,38], as well as at the pathological level in preclinical models [39]. In addition, mutations in several ALS-related genes, e.g., ANG and FUS [40-44], could provide a genetic link between ALS and frontotemporal dementia (FTD). Furthermore, C9orf72, TARDBP, and VCP can constitute a common genetic background among ALS and FTD, and, to a lesser extent, PD [42,45,46]. The analysis of the three “_Proteins” lists displayed a major overlap between PD and ALS compared to that of AD and ALS. Thus, the two motor disorders may share also common pathways of neurodegeneration, even though occurring in different cell types.

In order to identify PD-specific proteins, we eliminated all the proteins shared by AD and ALS and thus generated an only-PD_Proteins input list. Of these proteins, 25 were also found in the DisGeNET database. Probably they constitute major molecular hallmarks of PD, since they were never found to be altered in the other two pathologies and were reported both at the proteomic and the genetic levels. Many of them are ribosomal proteins, thus being implicated in protein synthesis. Another category over-represented in this group is the cellular response to stress. Eventually, TALDO1 was identified. Our group have already identified TALDO1 in T-lymphocytes, as part of a protein signature able to discriminate PD patients from control subjects and patients affected by atypical parkinsonism [47]. Therefore, this protein may mirror at the peripheral level an alteration occurring in the CNS [48]. Moreover, since this evidence was only based on one paper in the literature [48], we verified the up-regulation of the protein in 5 SN specimens of PD patients, with respect to 5 control subjects. As a proof-of-principle of our bioinformatics analysis, the increased level of the protein was confirmed in our samples. Although it is difficult to speculate about the link between a single protein and a complex, multifactorial disease, we may envisage a role of the pentose phosphate pathway in the regulation of mitochondrial homeostasis in the presence of enhanced oxidative stress [49]. Indeed, aberrant control of mitochondrial function has been reported in a cellular model that specifically recapitulates dopamine-linked oxidative stress [11].

Moving from protein identities to pathway analysis, we performed an ORA on the “_Genes” and “_Proteins” input lists, using the GO consortium, to highlight disease-specific processes. We expected that pathways shared by all three diseases were general hallmarks of cell death. Indeed, the ORA underlined the over-representation of the apoptotic process, which we used as a kind of positive control. Moreover, the ORA stressed the involvement of the Electron Transport Chain (ETC) in all three diseases. The respiratory chain, especially in pathological conditions, can be a source of reactive oxygen species (ROS). Markers of oxidative stress and ROS damage are elevated in PD, AD and ALS. A high level of ROS causes a damage to various cellular components and ultimately results in the activation of apoptosis [3]. Regarding the ALS specific-pathways, the involvement of RNA processing was particularly evident: metabolism (“rRNA metabolic process”, “ncRNA metabolic process”), transport (“Nuclear transport”, “Nuclear import” and “RNA transport”) and splicing (“mRNA splicing”) were all over-represented ontologies. Although Mitochondrial membrane organization and mitochondrial transport are involved in AD and PD, notably “Mitophagy” is over-represented only in PD. Alteration of the mitochondrial quality control and the consequent mitochondrial impairment have been widely associated to apoptosis directly triggered by these organelles (“Apoptotic mitochondrial changes”) [50]. The ORA of the only-PD_Proteins input list (i.e., proteins involved in PD never related to ALS and AD) again highlighted the importance of “Mitophagy”. This list gave us the opportunity to focus on proteins never associated to AD and ALS. At the same time, important pathways may be neglected due to the exclusion of too many members of the ontology under consideration. Keeping in mind advantages and shortcomings of this sub-list, we decided to perform the ORA using also a database of metabolic pathways, to evidence possible specific mechanisms. Some signal transduction cascades were suggested by the analysis. As an example, the Wnt signalling pathway emerged as a pathway commonly dysregulated in PD [51].

Eventually, we focused on protein complexes involved specifically in PD. The analysis of the PD interactor network evidenced four protein complexes: the proteasome 26S, the ETC complex

III, the CCT complex and the PPP2A complex. Focusing on these complexes, we decided to retrieve quantitative information from original papers. Since it was impossible to compare papers using different scales and normalization methods, we decided to categorize variations as up- or down-regulations. In the proteasome complex, it was possible to identify a different action of the PD pathogenetic process on the expression of members of the catalytic subunit (proteasome 20S) and of the 26S protease regulatory subunit. The formers were all up-regulated, whereas the latter were all down-regulated. PD has been considered for long time a proteasome disease [52] and our analysis seems to confirm this hypothesis. In the ETC complex III, proteins were reported to be regulated in different directions by the original papers, probably because of the use of different models [53]. This may indicate different alterations in the respiratory chain due to different perturbations. The involvement of the ETC dysfunction in PD has always been related to complex I [54] and the inhibition of this complex by MPTP is used to reproduce parkinsonism in animal models [55]. Nevertheless, considering all the results together allowed us to highlight also the role of complex III. The CCT complex has been demonstrated to play an active role in the protection of dopaminergic neurons [56], being composed by chaperonins. All the proteins of the CCT complex were reported as down-regulated in the papers considered in the present analysis, probably indicating an overwhelming of their re-folding/protective capacities. PPP2A is a protein complex composed by structural (A), regulatory/targeting (B) and catalytic (C) subunits. Some of these subunits are direct interactors of CCT complex chaperonins [57] and are implicated in many PD-related pathways. Indeed, this phosphatase controls Akt phosphorylation and thus macroautophagy [58], the activity of tyrosine hydroxylase (i.e., dopamine synthesis rate-limiting enzyme) [59] and alpha-synuclein aggregation [60].

To sum up, this analysis highlighted processes generically involved in neuronal death and pathways related to the loss of specific neuronal populations in the different diseases. In particular, it clarified cellular pathways specifically involved in PD. Moreover, the analysis highlighted the fundamental role played by four protein complexes in leading neurodegeneration in PD. This may

be useful for future research to better understand disease-specific mechanisms and general alterations related to neurodegeneration.

Conflict of interest

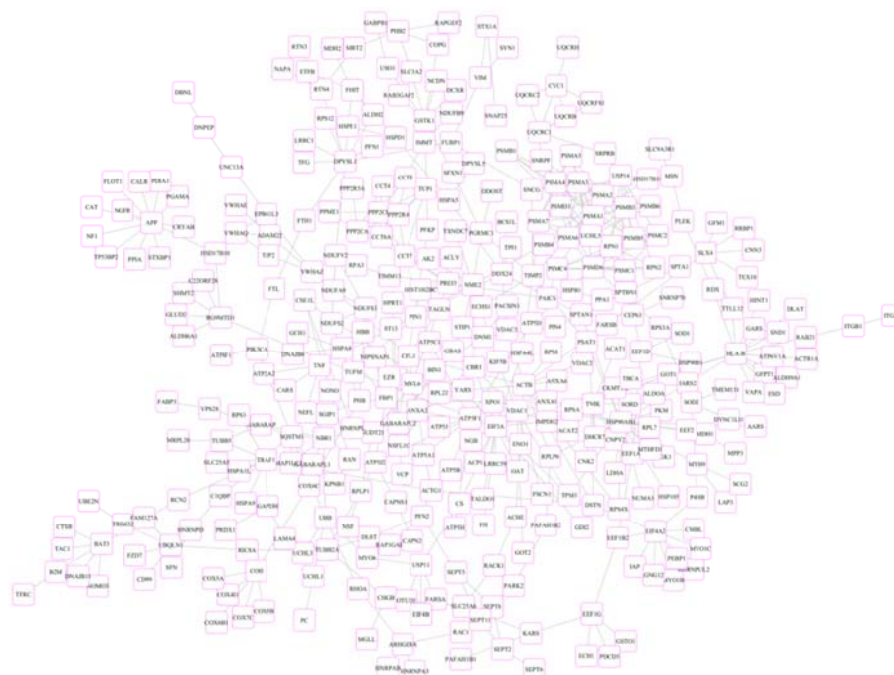
The authors declare that they have no conflict of interest.

Supplementary materials

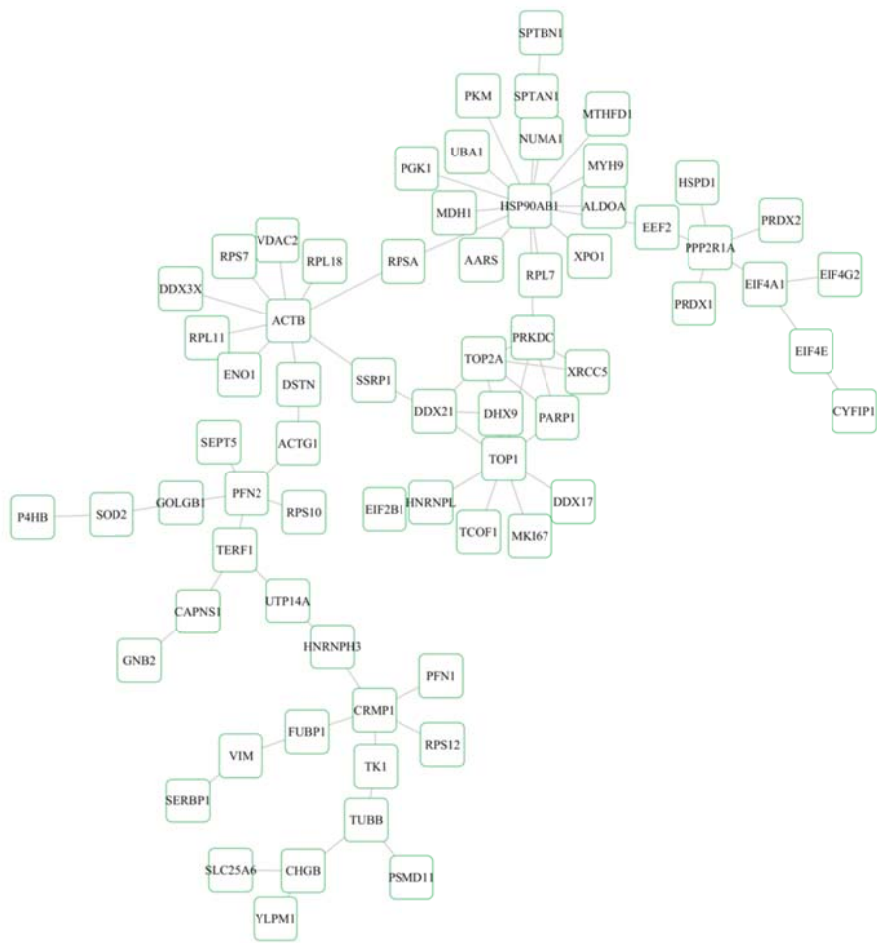
Supplementary Fig. 1: Network-based analysis of the “AD_Proteins” list (a), “PD_Proteins” list (b) and “ALS_Proteins” list (c), using the IntAct database as the reference set (PPI spider, $p < 0.01$).



a

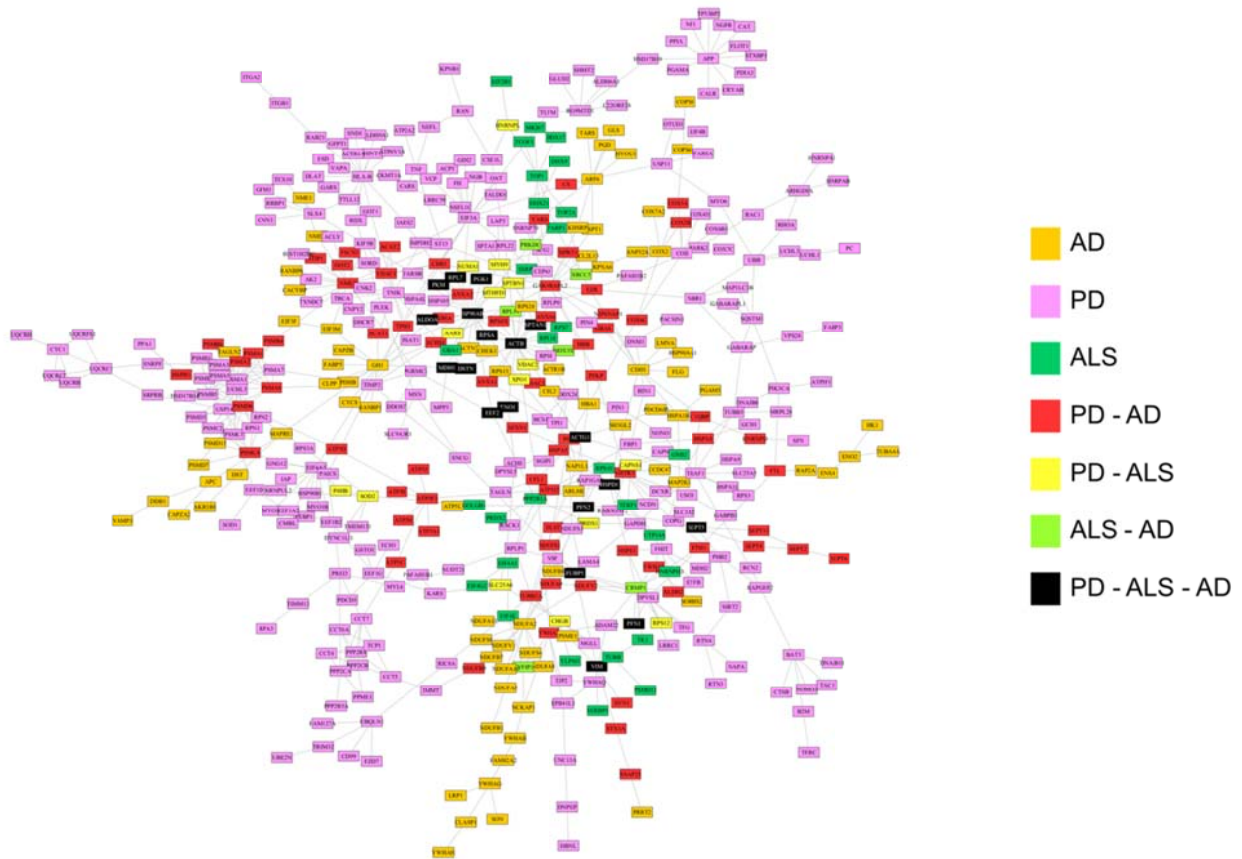


b



c

Supplementary Fig. 2: Network obtained by the union of AD, PD and ALS PPI networks (Supplementary Fig. 1). The colour code indicates disease-specific nodes and shared by different diseases.



Supplementary Table 1. Characteristics of PD patients and gender and age related control subjects (CTRL).

NBB number	Sex	Age	Diagnosis
2007-013	M	61	PD
2009-003	M	62	CTRL
2007-029	M	67	PD
1999-116	M	78	CTRL
2006-062	M	87	PD
2009-001	M	88	CTRL
2009-045	F	59	PD
2001-004	F	64	CTRL
2009-078	F	88	PD
1999-111	F	88	CTRL

Notes: NBB: Netherlands Brain Bank; M: Male, F: Female; CTRL: control subjects, PD:

Parkinson's disease

Supplementary Table 2. PMIDs of studies used to generate _Proteins lists.

	PMID
AD	11747211, 15756939, 15797529, 16519965, 17050040, 17111439, 17309106, 18648492, 18648646, 19241155, 20061648, 21237293, 21368863, 21388376, 21699958, 21883897, 21954051, 22559202, 22634250, 22926577, 23050487, 23154051, 23211594, 23231993, 23276639, 23391701, 23424162, 23457027, 23512986, 23537733, 23963966, 24306222, 24606058, 24893329, 25108202, 25457556, 25756589, 25818006, 25958317, 26059363
PD	15526345, 15755676, 15790536, 16150055, 16565515, 16889417, 17203978, 17490626, 17532186, 17705834, 18173235, 18226537, 18270577, 18338827, 18353766, 18384645, 18782562, 19498008, 19725078, 20155936, 20334438, 20403401, 20563739, 20594931, 20977677, 21136655, 21296869, 21322648, 21960009, 21988495, 22315971, 22410244, 22445325, 23562983, 24040246, 24449343, 24675778, 24737941, 24806433, 24834013, 24834013, 24841483, 25626353, 25683516, 25865804, 26468903
ALS	17979159, 17196550, 16847061, 15863242, 15501831, 19357085, 12475980, 25743254

Supplementary Table 3. Summary table of the most significant results of the GO Biological Processes and Reactome analysis of PD_Proteins-Genes input list.

PD_Proteins-Genes (25 IDs):	
Go Consortium Biological Process	Reactome
Alpha-amino acid metabolic process Cellular amino acid metabolic process Selenocysteine metabolic process Small molecule metabolic process	Cellular response to heat stress Metabolism of aminoacid and derivates

Supplementary Table 4. Summary of the protein level variations in PD complexes. ↓ indicates a down-regulation of the protein in PD, ↑ indicates up-regulation of proteins in PD.

	Gene Symbol	Expression	Pubmed ID	Model used
Respiratory chain	UQCRB	↓	24449343	Human SN
	UQCRC1	↓	24841483	Striatum in unilateral 6-OHDA-rat model
		↑	22445325	α-synuclein overexpression in SH-SY5Y cells
	UQCRC2	↓	22410244	Human SN
		↓	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (1 day)
		↑	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (7 days)
	UQCRH	↑	21960009	Nigrostriatal area in A53T and wt α-synuclein mice
		↓	15790536	SN in mice treated chronically with MPTP for 5 weeks
	UQCRSF1	↑	20403401	SN in 6-OHDA rat model
		↓	18270577	Striatum in MPTP macaque model
		↑	16565515	Human SN
	CYC1	↑	18173235	Striatum in MPTP and METH mice models
		↓	15790536	SN in mice treated chronically with MPTP for 5 weeks
	CCT complex	TCP1	↑	16150055
↑			25865804	mutant parkin (Q311R and A371T) overexpression in SH-SY5Y cells
CCT4		↑	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (1 day)
		↑	18173235	Striatum in MPTP mice model
		↑	18173235	Striatum in METH mice model
CCT5		↓	22445325	α-synuclein overexpression in SH-SY5Y cells
		↑	21322648	MPTP-treated N2a cells
		↓	15790536	SN in mice treated chronically with MPTP for 5 weeks
		↑	25626353	Whole brain in PINK1 knockout mice
CCT6A		↑	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (1 day)
CCT7		↑	22445325	α-synuclein overexpression in SH-SY5Y cells
	↑	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (1 day)	

	Gene Symbol	Expression	Pubmed ID	Model used
PPP2A complex	PPP2CA	↓	24449343	Human SN
	PPP2CB	↑	16150055	Cortical and striatal tissue in PARK2 knockout mice
		↑	26468903	α -synuclein fibrils-exposed SH-SY5Y cells
	PPP2R4	↓	20403401	SN in 6-OHDA rat model
		↑	20403401	SN in 6-OHDA rat model
PPP2R5A	↓	15790536	SN in mice treated chronically with MPTP for 5 weeks	
Proteasome complex	PSMB1	↑	21296869	Parkin-expressing SH-SY5Y cells treated with CCCP
	PSMB3	↑	21296869	Parkin-expressing SH-SY5Y cells treated with CCCP
	PSMB5	↑	21296869	Parkin-expressing SH-SY5Y cells treated with CCCP
		↑	16150055	Cortical and striatal tissue in PARK2 knockout mice
	PSMA1	↑	22315971	Co-expression of wt or mutated LRRK2 and Tau in <i>C. elegans</i>
		↑	21296869	Parkin-expressing SH-SY5Y cells treated with CCCP
	PSMA3	↑	21960009	Nigrostriatal area in A53T and wt α -synuclein mice
		↑	21296869	Parkin-expressing SH-SY5Y cells treated with CCCP
	PSMA7, PSMA5	↑	21296869	Parkin-expressing SH-SY5Y cells treated with CCCP
		↓	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (1 day)
	PSMC2	↓	20403401	SN in 6-OHDA rat model
		↓	22445325	α -synuclein overexpression in SH-SY5Y cells
	PSMC1	↓	15790536	SN in mice treated chronically with MPTP for 5 weeks
	PSMC3	↓	18338827	Ventral midbrain in MPTP-treated L1cam transgenic mice (1 day)
UCHL5	↓	17705834	Rotenone-treated MES cells	
USP14	↑	18173235	Striatum in MPTP and METH mice models	

6-OHDA: 6-hydroxydopamine, LRRK2: Leucine-rich repeat kinase 2, MES cells: dopaminergic neuronal cell line, METH: methamphetamine, MPTP:1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, N2a cells: murine neuroblastoma cell line, PARK2: Parkin gene, PINK1: PTEN-induced putative kinase 1, SH-SY5Y cells: human neuroblastoma cell line.

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