Monzón Sandoval, Jimena, Poggiolini, Ilaria, Ilmer, Tobias, Wade-Martins, Richard, Webber, Caleb ORCID: https://orcid.org/0000-0001-8063-7674 and Parkkinen, Laura 2020. Human specific transcriptome of ventral and dorsal midbrain dopamine neurons. Annals of Neurology 87 (6), pp. 853-868. 10.1002/ana.25719 file

Publishers page: http://dx.doi.org/10.1002/ana.25719
<http://dx.doi.org/10.1002/ana.25719>

Please note:
Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher’s version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See http://orca.cf.ac.uk/policies.html for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.
Objective: Neuronal loss in the substantia nigra pars compacta (SNpc) in Parkinson disease (PD) is not uniform, as dopamine neurons from the ventral tier are lost more rapidly than those of the dorsal tier. Identifying the intrinsic differences that account for this differential vulnerability may provide a key for developing new treatments for PD.

Methods: Here, we compared the RNA-sequenced transcriptomes of ~100 laser captured microdissected SNpc neurons from each tier from 7 healthy controls.

Results: Expression levels of dopaminergic markers were similar across the tiers, whereas markers specific to the neighboring ventral tegmental area were virtually undetected. After accounting for unwanted sources of variation, we identified 106 differentially expressed genes (DEGs) between the SNpc tiers. The genes higher in the dorsal/resistant SNpc tier neurons displayed coordinated patterns of expression across the human brain, their protein products had more interactions than expected by chance, and they demonstrated evidence of functional convergence. No significant shared functionality was found for genes higher in the ventral/vulnerable SNpc tier. Surprisingly but importantly, none of the identified DEGs was among the familial PD genes or genome-wide associated loci. Finally, we found some DEGs in opposite tier orientation between human and analogous mouse populations.

Interpretation: Our results highlight functional enrichments of vesicular trafficking, ion transport/homeostasis and oxidative stress genes showing higher expression in the resistant neurons of the SNpc dorsal tier. Furthermore, the comparison of gene expression variation in human and mouse SNpc populations strongly argues for the need of human-focused omics studies.
against the progressive motor symptoms associated with the loss of DA neurons.

DNA microarrays have been the most frequently used technique by which numerous gene expression studies have compared the postmortem brain tissue of PD patients to healthy controls, with varying results (see Zheng et al. for meta-analysis). The majority of these studies have, however, used SNpc bulk tissue, where the subpopulations are not identified and the cellular composition is affected by the neurodegenerative process itself, including reactive astrogliosis and microglial activation. Their weakness is that they ultimately focus on downstream consequences of the disease process, rather than the underlying causes. Analysis of cell-type-specific gene expression is possible with laser capture microscopy that allows the isolation of precise neuronal populations from the tissue. However, dissecting the rare surviving DA SNpc neurons remaining in end-stage PD may only select cells more resistant to the disease process rather than revealing clues to the initial neurodegenerative mechanisms. Several studies have also compared transcriptomic profiles of SNpc and ventral tegmental area (VTA) in rodents but how they relate to human gene expression is unknown. Up to 6 molecularly distinct subpopulations of SNpc and VTA were identified in mice by single-cell gene-expression profiling using microfluidic dynamic array on fluorescence-activated cell sorting (FACS)-sorted midbrain DA cells. Recently, these mouse subpopulations were also confirmed by RNA-sequencing (RNA-seq), which is superior to microarrays in both sensitivity and coverage.

In the present study, for the first time in humans, we compared the RNA-seq–derived transcriptomes of laser capture microdissected (LCM) ventral (vulnerable) and dorsal (resistant) tier neurons of the SNpc from healthy individuals, to gain insight into the intrinsic differences between these neuronal populations that could explain their differential vulnerability in disease. Understanding the molecular complexity of healthy midbrain DA neurons will help uncover novel candidates for a new generation of targeted therapeutic approaches to PD.

**Subjects and Methods**

### Sample Collection and Preparation

We extracted RNA from the frozen midbrain of ~30 healthy controls obtained from the Oxford MRC Control Brain Bank, South Central–Oxford C Research Ethics Committee approved the study (ethical license REC15/SC/0639). The RNA integrity number (RIN) was analyzed in these samples using Bioanalyzer (Agilent Technologies, Santa Clara, CA) and ranged between 6.6 and 9.2. Only 7 healthy controls with the highest RIN scores (>8) were selected for the further analysis. The mean age at death in these 7 healthy controls (2 female, 5 male) was 70.7 ± 12.3 years (range = 56–93 years). Briefly, 10μm-thick sections of the frozen midbrain were cut at the level of the 3rd nerve, dehydrated in ethanol series, and stained with cresyl violet in strictly RNA-free conditions. Single neuromelanized neurons were isolated from separate, nonoverlapping ventral and dorsal tiers of SNpc using anatomic criteria and harvested from the stained cryosections by using a PALM Robot–Microbeam system (Carl Zeiss, Oberkochen, Germany; Fig 1).

### RNA Extraction and Sequencing Processing

In 7 healthy controls, the total RNA was extracted from ~100 pooled LCM neurons from each tier using PicoPure RNA isolation Kit (Arcturus, Mountain View, CA) with DNase digestion step (RNase-Free DNase Set; QIAGEN, Hilden, Germany). The cDNA was prepared for the sequencing using the SMARTer Kit (Clontech Laboratories, Mountain View, CA). A total of 14 samples, 2 from each tier of 7 healthy controls, were sequenced (HiSeq4000 75bp paired-end) across 2 lanes. The resulting reads were mapped to cDNA sequences from *Homo sapiens* GRCh38 release 85 available through the Ensembl FTP site (ftp://ftp.ensembl.org/pub/). Kallisto version 0.42.4 was used to create an index and estimate counts and transcript abundances; default parameters were used except for the number of bootstraps, which was set to 100. Finally, the tximport function in R was used to summarize transcript abundances for all protein-coding genes.

### Differential Expression

We used removal of unwanted variance (RUV) analysis to identify unwanted sources of variation. The first factor identified by RUV was found across a set of 1,000 empirical control genes and was found to be strongly correlated with the RIN score (r = 0.956). We incorporated the first factor identified into the model to test for differential expression between ventral and dorsal neurons using DESeq2. The model also considered the origin of the samples (ie, brain donor). Default filtering option was set up, and those genes with either a lower number of counts or outliers were filtered out. After filtering, 14,033 remained, and those were used as our background gene population for further analysis. Genes with a false discovery rate (FDR) < 0.05 were considered differentially expressed.

### TaqMan Reverse Transcribed Polymerase Chain Reaction Assay Validation

About 100 SNpc neurons were LCM in triplicates from each tier from the same healthy controls used for the RNA-seq analysis. The total RNA was extracted using PicoPureTM RNA Isolation Kit (Applied Biosystems, Foster City, CA) from 3 LCM preparations for each tier per case and pooled during elution phase. This step was carried out to gain at least ~50ng of RNA, which was then reverse transcribed using SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA). The obtained cDNA was assayed on a Rotor-Gene Q Real-Time PCR System (QIAGEN) using TaqMan Gene Expression Assays (Life Technologies, Carlsbad, CA) by following standard protocols (Table). Reactions were performed in duplicates using the FAM dye-labeled assay. No-template controls were run to determine any contamination.
Amplification for TaqMan probes reactions was performed in a 20μl reaction volume, using 10μl TaqMan Universal Master Mix 2X (Applied Biosystems), 2μl cDNA, and 1μl TaqMan Probe 20x. The comparative delta Ct method (also known as the 2-ΔΔCt method) was used to calculate the relative fold change in gene expression. Data were normalized to housekeeping genes (GAPDH and B2M) and gene expression in the ventral tier of SNpc (used as control = 1).

**Immunohistochemistry and Immunoblotting**

Six-micrometer-thick sections of the midbrain depicting SNpc on the level of the 3rd nerve were cut from the age-matched healthy controls (n = 5) and processed for immunohistochemistry. Following antigen retrieval with autoclave at 120°C for 10 minutes in citric buffer pH 6.0, endogenous peroxidase activity was eliminated with treatment with 3% H2O2 (in phosphate-buffered saline) and PCP4, BAB3B, and HCN1 antibodies (dilution 1:500, see above for details) incubated overnight at 4°C. For detection, the REAL EnVision Detection System (Dako, Carpinteria, CA) was used, with diaminobenzidine as chromogen.

Two 2mm-diameter punctures were taken from the frozen SN, 1 from the ventral and 1 from the dorsal tier, from the same healthy controls used for the RNA-seq analysis. In addition, we dissected 2mm-diameter punctures from the dorsal SNpc from 3 cases with incidental Lewy body disease (with Braak PD stages I–III) and 3 PD patients (with Braak PD stages V–VI). Tissue was homogenized in lysis buffer (5mM hydroxyethylpiperazine ethane sulfonic acid, pH 7.4; 320mM sucrose; 1mM ethylenediaminetetraacetic acid; 0.1% sodium dodecyl sulfate; and protease inhibitors and phosphatase inhibitors [Roche, Basel, Switzerland]) to 6% wt/vol. Following incubation on ice for 30 minutes, the homogenates were spun at 5,000rpm for 10 minutes at 4°C. The total protein amount was determined using the BCA Protein Quantification Kit (Abcam, Carpinteria, CA). (FIGURE 1: Protocol of workflow and quality control. (A) Protocol of workflow: from each healthy control donor, approximately ~100 neurons from the ventral (Ve) and dorsal (Do) tier of the substantia nigra (SN) were laser capture microdissected (LCM). Cells from 1 tier in each donor were pooled, RNA was extracted, and cDNA was obtained and prepared for sequencing, allowing us to compare the gene expression in vulnerable versus resistant neuronal populations and finally to perform functional analysis. 3rd = oculomotor nerve; GO = Gene Ontology; MP = Mammalian Phenotype; PL = pars lateralis. (B) Mapping rates as the percentage of reads mapping protein-coding genes in each of the 14 available samples. The dashed line at 30% has been drawn to highlight samples with particularly lower mapping rates (healthy control HC1, 3, and 4 were excluded from further analysis). (C) Library complexity shown as the cumulative proportion of the library for the top 100 most expressed features/genes in each sample. (D) First 2 principal components (PC), which together explain ~45% of the variance. Label colors reflect the origin of the samples, whereas the intensity of the dots denotes the total number of counts in each sample (darker colors represent higher number of counts).
Cambridge, UK) and adjusted to 2mg/ml. Brain samples were diluted with 0.1× sample buffer followed by 4 parts of diluted sample combined with 1 part of Fluorescent Master Mix and heated at 95°C for 5 minutes. The denatured samples, blocking reagent, primary antibodies (PCP4, 14705-1-AP, Proteintech Group, Rosemont, IL; RAB3B, 15774-1-AP, Proteintech Group; HCN1, GTX131334, GeneTex, Irvine, CA, all with 1:50 dilution; and β-actin ab8224, Abcam, 1:100 dilution), mouse and rabbit horseradish peroxidase–conjugated secondary antibodies, and chemiluminescent substrate were dispensed into a 384-well plate. A biotinylated ladder provided molecular weight standards for each assay. After plate loading, the separation electrophoresis and immunodetection steps took place in the fully automated Peggy Sue (Protein Simple, San Jose, CA) capillary system.

**Functional Enrichment Analysis**

Gene Ontology (GO) annotations were obtained through biomaRt in R from Ensembl release 85, whereas Mammalian Phenotype (MP) annotations were retrieved from the MGI Batch Query tool (accessed on December 16, 2016) using all available Ensembl gene IDs from Mus musculus with 1-to-1 ortholog correspondence to Homo sapiens. GO enrichment analysis included terms with at least 50 genes in our background population. MP enrichment analysis included all 28 major phenotypes first, and if a major phenotypic term was significantly enriched, we performed a second enrichment analysis among more specific phenotypic terms (with a minimum of 20 genes in our background population). For the actual test, we compared the number of genes assigned to each term to an estimated expected number derived from 1,000 equally sized random samples of genes. A p value has been estimated based on a z score derived from the randomizations and further adjusted for multiple testing using the Benjamini–Hochberg method. GO terms with an FDR < 0.05 and containing at least 2 genes were deemed significantly overrepresented.

**Coexpression**

Based on the expression profiles across all samples available from multiple brain structures across development in BrainSpan, we calculated the coexpression between each gene pair as the Pearson correlation between their expression levels. The average coexpression between all gene pairs in each subset of differentially expressed genes (DEGs) was measured and compared to 10,000 equally sized random samples of genes. A p value has been estimated based on a z score derived from the randomizations and further adjusted for multiple testing using the Benjamini–Hochberg method. GO terms with an FDR < 0.05 and containing at least 2 genes were deemed significantly overrepresented.

---

**TABLE. Validation of Gene Expression Using TaqMan Real-Time Polymerase Chain Reaction**

| Gene   | Protein                                                               | TaqMan Assay ID          |
|--------|-----------------------------------------------------------------------|--------------------------|
| GAPDH  | Glyceraldehyde-3-phosphate dehydrogenase                              | Hs009999905_m1           |
| B2M    | Beta-2-microglobulin                                                  | Hs009999907_m1           |
| TH     | Tyrosine hydroxylase                                                 | Hs00165941_m1            |
| DAT    | Dopamine transporter                                                 | Hs00997374_m1            |
| VMAT2  | Vesicular monoamine transporter 2                                     | Hs00996834_g1            |
| GFAP   | Glial fibrillary acidic protein                                       | Hs00909233_m1            |
| PMP22  | Peripheral myelin protein 22                                          | Hs00165556_m1            |
| GAD1   | Glutamate decarboxylase 1                                            | Hs01065892_m1            |
| PCP4   | Purkinje cell protein 4                                               | Hs01113637_m1            |
| RAB3B  | Ras-related protein Rab-3B                                            | Hs01001137_m1            |
| HCN1   | Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 | Hs01570432_m1 |
| SPARC  | Secreted protein acidic and rich in cysteine                          | Hs00234160_m1            |
| SNX8   | Sorting nexin 8                                                       | Hs01030705_m1            |
| MT1G   | Metallothionein-1G                                                   | Hs01584215_g1            |
| ANXA1  | Annexin A1                                                           | Hs00167549_m1            |
| ATP13A4| Cation-transporting ATPase 13A4                                      | Hs01115518_m1            |
| LYPD1  | Ly6/PLAUR domain-containing protein 1                                 | Hs00375992_m1            |

---

856 Volume 87, No. 6
Protein–Protein Interactions
A combined protein–protein interaction network was created based on diverse resources: BioGRID 3.4 (accessed on October 13, 2016),22 HitPredict (accessed on October 20, 2016), IntAct (accessed on October 11, 2016), STRING (accessed on November 29, 2016, restricted to *H. sapiens* and experimental scores >0), CORUM (accessed on October 11, 2016), and Reactome (accessed on October 11, 2016). The combined network reflected the presence of an interaction in any of the aforementioned resources, representing a total of 20,591 genes and 1,973,967 interactions, where self-interactions were not considered. We have measured both the number of interactions and the clustering in the combined protein–protein interaction network among DEGs between ventral and dorsal neurons. To compare the observed parameters to those expected by chance, we have created 1,000 equally sized randomizations with a similar degree distribution to set of DEGs. To accomplish this, we have log2-transformed the degree distribution of all genes in our background population, divided all genes into 20 bins, and counted the number of DEGs falling in each bin, so for each randomization an equal number of genes from each bin would be drawn. Finally, a p value would be estimated based on the number of cases in which the number of interactions of the clustering was either equal to or higher than that observed for each set of DEGs.

Phenotypic-Linkage Network
A phenotypic-linkage network (PLN) has been used to integrate diverse functional sources of information into a single measure. From coexpression based on the BrainSpan dataset, only the top 5% of the strongest correlations were taken into account for the PLN. The combined protein–protein interaction network has also been included, as well as similarity between shared GO annotations, measured separately for biological processes, molecular function, and cellular component.23

Results
Our study sought to reveal the intrinsic differences in gene expression patterns in the human healthy control individuals between the 2 neuronal populations, ventral and dorsal SNpc, known to show selective vulnerability in PD. For this, we compared the transcriptional profiles of ventral and dorsal SNpc neurons obtained from the midbrain at the level of the 3rd nerve from 7 healthy brain donors. Approximately ~100 cells from each tier were LCM, followed by RNA extraction and sequencing of each sample (see Fig 1). Samples from 3 of 7 donors were removed from further analysis, given their particularly low mapping rates and reduced library complexity. Principal component analysis based across all sequenced samples segregated along the first component samples identified and removed by their low quality.

Transcriptional Signature of DA Neurons
We confirmed the DA identity of our samples (Fig 2A) by looking at the transcript levels of dopamine transporters (*VMAT* and *DAT*), enzymes involved in the synthesis of dopamine (*TH* and *DDC*), and transcription factors involved in dopamine specification and maintenance.

![Figure 2: Characterization of laser capture microdissected (LCM) dopaminergic neurons.](image)

A. Samples showed high gene expression levels (log2(CPM + 1) (CPM means counts per million)) of dopaminergic markers including dopamine transporters, enzymes involved in dopamine synthesis, and transcription factors necessary for dopaminergic specification, whereas ventral tegmental area–specific markers (*ADCYAP1, LPL, OTX2*, and VIP) were virtually absent. The lines denote the mean and median expression of the whole background gene population. (B) Assessment of dopamine neuron sample enrichment and purity by reverse transcribed quantitative polymerase chain reaction in LCM substantia nigra pars compacta (SNpc) neurons in relation to whole midbrain sections that have not been altered in terms of cellular content and therefore contain dopamine neurons and many other cell types. The 2-ΔΔCt method shows increased expression of the dopaminergic genes DAT, TH, and VMAT2 in the LCM SNpc neurons normalized to sections (value 1, red line), indicating dopamine neuron enrichment. A significant reduction in expression of genes for the glial fibrillary acidic protein 1 (GFAP), peripheral myelin protein 22 (PMP22) and glutamate decarboxylase 1 (GAD1) suggests isolation of a relatively pure population of dopamine neurons. (C) Proportion of neurons, astrocytes, microglia, oligodendrocytes, oligodendrocyte precursor cells (OPC), and endothelial cells estimated by MuSiC, an RNA-sequencing deconvolution method.
followed by differential gene expression analysis. Among chance by permuting label groups (dorsal/ventral) of samples whether the number of DEGs was higher than expected by
expressed (FDR < 0.05), 58 of which were higher in the dor-
sal samples. In total, 106 genes were detected as differentially
gene expression variance (Fig 3A). We then applied DESeq2
along the 5th component, which accounts for 2.44% of the
source (genotype) variation rather than anatomical localiza-
data continued to be associated with technical and sample
application of RUV, the principal drivers of variation in the
microglia, and endothelial cells. RNA-seq that we applied along with single cell gene expres-
with RIN score (Pearson correlation

to remove the
(see Subjects and Methods). We
lowed at later Braak stages (V
remor el i ke t o b ei n v o l ve d i n a si m i la rf u n c t i o n ,
spondingly, the expression of astroglial (GFAP), oligoden-
droglia (PMP22), and γ-aminobutyric acidergic (GABAergic; GAD1) genes were very low, suggesting isolation of a rela-
tively pure population of dopamine neurons. Finally, the propor-
tion of brain cell types within our samples was estimated
with MuSiC, a recent deconvolution method for bulk
RNA-seq that we applied along with single cell gene expression
data from neurons, oligodendrocytes, astrocytes,
microglia, and endothelial cells. This estimate supported
a very high percentage of neurons within our samples
(mean = 96.87%; see Fig 2C). No significant difference was
found between dorsal and ventral samples (p > 0.05).

**DEGs between Human Ventral and Dorsal SNpc Neurons**

To obtain an unbiased set of genes with distinct patterns of expression between human ventral and dorsal SNpc neurons, we considered 14,033 protein-coding genes remaining following the removal of outliers and genes with low expression (see Subjects and Methods). We first applied RUV analysis to remove the first factor of variation, which was associated with RIN score (Pearson correlation r = 0.956). Despite application of RUV, the principal drivers of variation in the data continued to be associated with technical and sample source (genotype) variation rather than anatomical localization. Nonetheless, ventral and dorsal samples segregated along the 5th component, which accounts for 2.44% of the gene expression variance (Fig 3A). We then applied DESeq2 accounting for the origin (donor) of each pair of ventral/dorsal samples. In total, 106 genes were detected as differentially expressed (FDR < 0.05), 58 of which were higher in the dorsal/resistant neurons, whereas the remaining 48 were higher in the ventral/vulnerable neurons (see Fig 3B, C). We tested whether the number of DEGs was higher than expected by chance by permuting label groups (dorsal/ventral) of samples followed by differential gene expression analysis. Among these permutations, the number of genes detected as differentially expressed was always smaller than the number obtained when the correct labels were assigned (observed = 106, permutations = 41, 67, and 25), confirming that the number of DEGs identified between tiers was higher than expected by chance.

**Validation with RT-qPCR and Further Protein Analyses of the Top DEGs**

Using the 2-ΔΔCt method to determine the fold differences of relative gene expression between dorsal and ventral SNpc tiers in our top 10 DEGs largely confirmed the results from the RNA-seq analysis (Fig 4). As predicted, RT-qPCR analysis found PCP4 highly increased in dorsal versus ventral SNpc neurons, followed by RAB3B, HCN1, SPARC, SNX8, and MT1G, whereas the expression of ATP13A4 and LYPD1 were decreased. However, we could not confirm by RT-qPCR the dorsal upregulation of GFAP and ANXA1 found by RNA-seq. The PCP4 (Purkinje cell protein) immunohistochemistry showed extensive labeling of the processes in the SNpc and pars reticulata. However, we could not detect any cytoplasmic staining of the pigmented DA SNpc neurons. The RAB3B expression was also rather diffuse throughout the midbrain, whereas after trialing several HCN1 antibodies we could not detect any reliable staining in our postmortem brain tissue. Using automated quantitative Western blot, we further examined the protein levels of our top 3 DEGs in dorsal versus ventral SNpc tier (in 2mm-diameter punctures). We found that PCP4 and HCN1 appeared to have slightly higher protein levels in the dorsal SNpc tier, similarly to their gene expression, whereas RAB3B showed higher protein levels in the ventral SNpc, in contrast to the mRNA level findings. However, the variability between punctured samples was high, and none of the differences reached statistical significance. Furthermore, we examined protein expression levels of the same 3 proteins in dorsal SNpc during the pathological progression of PD and showed that HCN1 protein expression significantly increased with Braak PD stages (analysis of variance, p = 0.03). The PCP4 levels appeared to increase at later Braak stages (V–VI), whereas RAB3B levels were more stable throughout the disease progression.

**Insights from Functional Analysis**

As a general approach, we looked for evidence of functional associations among each group of upregulated genes (ventral/vulnerable and dorsal/resistant neurons), while controlling for the total set of genes expressed across the sampled cells. Gene pairs with coordinated expression patterns are more likely to be involved in a similar function, and so we determined the coordinated expression pattern for each pair of DEGs with the brain across different structures and
FIGURE 3: Gene expression differences between human vulnerable and resistant substantia nigra pars compacta (SNpc) neurons. 
(A) Principal component (PC) 5 after removal of unwanted variance factor 1 separates ventral and dorsal SNpc neurons and explains ~2.44% of the variance. HC = healthy control. (B, C) Expression patterns for genes detected differentially expressed between tiers (false discovery rate < 0.05) shown for (B) genes with significantly higher expression in ventral/vulnerable SNpc and (C) genes with significantly higher expression in dorsal/resistant SNpc. Gene expression is given in counts per million (CPM) and scaled by gene.
development using the BrainSpan Atlas of the Developing Human Brain. We found that the average coexpression between increased genes in the dorsal/resistant neurons is higher than we would expect from equally sized random samples of genes (empirical $p = 0.0005$), supporting convergent functionality. However, genes increased in the ventral/vulnerable neurons are not significantly coexpressed across brain tissues (empirical $p = 0.2762$).

Using a comprehensive integrated functional genomics approach, termed a PLN, we looked for evidence of functional convergence among DEGs. A PLN combines multiple sources of functional information (GO annotations, coexpression, and protein–protein interactions) into a single weighted measure of gene functional relatedness between all pairs of genes.

To measure functional convergence, we compared the sum of the weighted edges between gene pairs in the PLN. We observed higher than expected functional convergence between genes increased in the dorsal/resistant neurons ($p < 0.001$; Fig 5A), whereas genes increased in the ventral/vulnerable neurons did not functionally converge more than expected by chance ($p = 0.594$; see Fig 5B). When considering the structure of links among DEGs within the PLN by calculating the global clustering and in comparison to random samples of genes, we noticed a higher than expected clustering for both increased genes in the dorsal/resistant ($p = 0.033$) and in the ventral/vulnerable neurons ($p = 0.004$). Overall, genes increased in the dorsal/resistant neurons have stronger links and tend to share links with neighboring genes within the
PLN, whereas genes increased in the ventral/vulnerable neurons have weaker links but still share common neighbors within the PLN. Taken together, these network results provide evidence of functional clustering and thus shared functionality.

Among genes higher in the dorsal/resistant neurons, we found significantly overrepresented GO Biological Process terms: receptor mediated endocytosis (fold overrepresentation \([\text{FOR}] = 8.62\)), calcium ion homeostasis (\(\text{FOR} = 7.49\)), potassium ion transport (\(\text{FOR} = 7.43\)) regulation of cell proliferation (\(\text{FOR} = 7.56\)), cell surface receptor signalling (\(\text{FOR} = 4.53\)), central nervous system development (\(\text{FOR} = 7.69\)), cellular response to TNF (\(\text{FOR} = 8.62\)), and insulin stimulus (\(\text{FOR} = 7.66\); see Fig 5C). By contrast, genes higher in ventral/vulnerable neurons were overrepresented in processes such as the negative regulation of cell migration (\(\text{FOR} = 14.15\)), canonical Wnt signalling (\(\text{FOR} = 11.49\)), and positive regulation of proteasomal ubiquitin dependent protein catabolic process (\(\text{FOR} = 9.47\); see Fig 5D). The complete GO enrichment analysis results are

**FIGURE 5:** Functional convergence among increased genes in neuronal populations of each substantia nigra pars compacta (SNpc) tier. The phenotypic linkage network integrates diverse sources of functional association (gene ontology, coexpression, and protein–protein interactions) into a single measure of functional similarity between genes, where nodes represent genes and edges denote the weighted functional association between them. (A, B) Phenotypic linkage subnetwork for all increased genes (A) in the dorsal/resistant neurons and (B) in the ventral/vulnerable neurons. (C, D) Overrepresented gene ontology annotations (false discovery rate \([\text{FDR}] < 0.05\)) for (C) genes with higher expression in dorsal/resistant SNpc and (D) for genes with higher expression in ventral/resistant SNpc. Dashed lines indicate an FDR threshold of 0.05.
given in the Supplementary Table. Overrepresentation of a unique set of specific processes was found among genes upregulated in neurons from each tier.

Additionally, we looked for any unusually overrepresented phenotypes among the unique (1:1) orthologues of these genes that had been experimentally disrupted in the mouse. Among genes increased in the dorsal/resistant neurons, we found 4 phenotypic classes overrepresented (Fig 6A), including Nervous System (FOR = 1.84) and Homeostasis/Metabolism (FOR = 1.79). Examining more specific terms within the Nervous System category (see Fig 6B) revealed an overrepresentation of genes whose unique mouse orthologue’s disruption yields a decreased nerve conduction velocity (FOR = 22.38), abnormal myelination (FOR = 9.64), and axon degeneration (FOR = 7.75). No mouse orthologue phenotypic associations were found among genes increased in the ventral/vulnerable neurons.

**Links to Known PD Genes**

The differential susceptibility to neurodegeneration between neurons from the ventral and dorsal tiers of SNpc led us to search for genes (and interactions) linked to the disease in the DEG sets. We examined the identified DEGs between the 2 neuronal populations for genes associated with familial PD, or those genes located within regions of high linkage disequilibrium (LD) to single nucleotide polymorphisms.

---

**FIGURE 6:** Mammalian Phenotype annotation and overrepresentation analysis for increased genes in the dorsal/resistant substantia nigra pars compacta (SNpc) neurons. (A) Genes with higher expression in dorsal/resistant neurons annotated to at least 1 overrepresented Mammalian Phenotype class (false discovery rate < 0.05). (B) Specific nervous system phenotypes overrepresented in genes with higher expression in dorsal/resistant SNpc neurons. (C) Specific homeostasis- or metabolism-associated phenotypes overrepresented in genes with higher expression in dorsal/resistant SNpc neurons. CNS = central nervous system.
FIGURE 7: Human gene expression patterns in ventral and dorsal substantia nigra pars compacta (SNpc) neurons. (A) Genes that were expected to have higher expression in the ventral tier of SNpc according to previous mouse data also included genes (SATB1, ANXA1, FGF1, and PTPN5) that showed higher expression in the dorsal tier of SNpc in humans. (B) Likewise, genes that were expected to have higher expression in the dorsal tier of SNpc also included LYDP1, with higher expression in the ventral tier of SNpc in humans. CPM means Counts per million.
(SNPs) associated with the disease by recent PD meta-
yses.18 None of the DEGs was among the familial PD 
genes or in high LD to associated genome-wide association 
study (GWAS) SNPs. Although we did identify protein– 
protein interactions between DEGs and PD-associated 
genes, the number of interactions was no more than we 
would expect by chance when compared to random genes 
expressed in these neuron types.

**Human to Mouse Gene Expression Comparison**

Mouse FACS-sorted midbrain DA neurons have been pre-
viously distinguished based on the transcriptional profiling 
with an array including genes reported to be differentially 
expressed between SN and VTA, validated DA markers, 
and housekeeping genes.14 Two DA neuron clusters identi-
fied by Poulin et al14 had similar expression profiles to 
SNpc (DA1A-1B) and VTA (DA2A-2D), and further sub-
division into DA1A and DA1B subtypes corresponded to 
the ventral and dorsal tiers of human SNpc, respectively. 
Furthermore, recently mouse DA neurons were classified 
into 5 distinct subpopulations DA-SNC and DA-VTA1–4 
using single-cell RNA-seq.15 As the DA-VTA1 subgroup 
in La Manno et al15 was positioned more toward the dorsal 
SNpc per Allen Mouse Brain Atlas, we considered this 
comparable to our human dorsal SNpc and the mouse 
DA-SNC subgroup to be comparable to our human ventral 
SNpc. Thus, for gene markers reported to be different 
between DA1A/DA-SNC and DA1B/DA-VTA1 in mice, we 
looked at the relative expression in our human data. 
Some genes reported to have higher expression levels in anal-
ogous ventral SNpc mouse subpopulations (ALDH1A1, 
SNCA, SOX6, GRIK1, and NOSTRIN) showed similar tier-
specific directionality in humans (Fig 7A), although none of 
the human genes reached statistical significance. Similarly, 
CHRNA4, FJK1, FOXA2, CALB1, GFRA2, and POUSF1, 
with higher reported expression in analogous dorsal SNpc 
mouse subpopulations, also tended to have higher levels in 
our human dorsal SNpc neurons (see Fig 7B). However, we 
also identified genes with opposite trends between the 
species. For example, SATB1, FGF1, PTPN5, and ANXA1 
were unexpectedly more expressed in the human dorsal 
SNpc neurons and LYPD1 in the ventral SNpc neurons, 
despite the opposite direction having been reported in 
mice.14,15 Nevertheless, from all the genes compared 
between humans and mice, only 2 (ANXA1 and LYPD1) 
were deemed differentially expressed in our human analysis 
(FDR < 0.05).

**Discussion**

Our study focused on comparing the RNA-seq trans-
criptomic profiles of the ventral and dorsal tiers of SNpc 
neurons in healthy individuals as we sought to identify 
intrinsic differences between the 2 neuronal populations 
that could inform on their differential vulnerability in the 
context of PD. Performing the comparison among healthy 
individuals aids in capturing similar numbers of neurons 
from both tiers; furthermore, the selection of cells is not 
biased toward only surviving neurons, as might occur if 
the same neuronal populations were compared between 
PD patients and healthy controls. LCM neurons were 
selected on the basis of their pigmentation and anatomic 
localization and expressed very high levels of VMAT, 
DAT, TH, and DDC genes, reflecting a DA profile. No 
significant differences in these DA marker gene levels were 
found between neurons from the ventral and dorsal tiers 
of the SNpc, supporting our aim to capture and compare 
similar numbers of DA neurons. It is also important that 
the expression levels of genes reported to be specific to the 
VTA (OTX2, ADCYAPI, and VIP) were virtually undetectable, reflecting the absence of VTA DA neurons 
in our samples. In addition to RNA-seq, we validated the 
level of enrichment and purity of dopamine neurons by 
RT-qPCR, assessing the dopamine neuron and glial/ 
GABAergic-specific gene expression in the LCM cells ver-
sus whole midbrain sections. However, despite the careful 
execution of LCM, "contamination" with non-neuronal 
transcription signals (eg, GFAP, PMP22) can occur, but 
this is at much lower levels than what is found in the bulk 
tissue. However, we also confirmed a very high neuronal 
proportion of our sample by MuSiC,24,25 a recent RNA-
seq deconvolution method for bulk tissue.

We found 106 DEGs between neuronal populations 
from the ventral and dorsal tier (FDR < 0.05), 
corresponding to 0.75% of our background gene population 
and 0.5% of all protein-coding genes. We consider that 
some genes increased in the dorsal neurons (n = 58) 
could provide a protective effect in PD, and/or conversely, 
genes increased in the ventral neurons (n = 48) might con-
fer higher vulnerability. Of particular interest in the con-
text of neurodegeneration and PD are those genes that 
show abnormal phenotypes of the nervous system when 
the corresponding mice ortholog is disrupted, but also 
those directly involved in metabolic processes, given the 
high energy demand of DA neurons. Our study revealed a 
coherent group of genes increased among the dorsal/resis-
tant neurons with functional convergence in phenotypes 
associated with neural function and homeostasis, many of 
which may be relevant to the possible protective effect of 
these neuronal populations in PD (see below). These genes 
demonstrated more coordinated temporal and spatial 
expression patterns across the human brain, their protein 
products were more likely to interact, and they 
presented stronger functional associations with each other 
than expected by chance in an integrative functional gene
network. In contrast, genes increased in the more vulnerable ventral SN neurons did not exhibit strong evidence for shared functionality.

**Vesicular Trafficking**

Genetic discoveries have brought defects in vesicle trafficking to the forefront of potential pathogenic players in PD. The Rab protein family enriched in neuronal synaptic vesicles plays a key regulatory role in vesicle trafficking. Rabs have been shown to be closely associated with α-synuclein (aSyn)-mediated pathological processes but also to interact with many PD-related genes, and thus they could plausibly be regarded as novel biomarkers or therapeutic targets. We found 2 members of this family increased among the dorsal/resistant SNpc neurons: RAB3B and RAB13. The subfamily of RAB3 proteins in particular has been reported to have stronger gene expression in the relatively spared VTA than in the SN neurons in a human brain and elevated protein expression in the projections of the VTA in the ventromedial striatum of the rat. When overexpressed in rat SN DA neurons, it increases the number and size of synaptic vesicles in the DA presynaptic terminals and dopamine content in the striatum. When RAB3B is overexpressed, it also shows a protective effect in DA neurons treated with oxidative stressor 6-hydroxydopamine and the proteasome inhibitor. Conversely, when RAB3B is knocked down by siRNA, DA neurons show an increased vulnerability to both toxins. A protective effect of RAB13 on the other hand involves the clearance of aSyn inclusions through endocytic recycling and rescued aSyn-induced toxicity. Furthermore, several members of the RAB family including RAB3 get phosphorylated by LRRK2, whereas RAB13 phosphorylation occurs in response to PINK1 activation.

In addition to Rab genes, SNX8 (sorting nexin 8) and ANXA1 (annexin A1), genes involved in intracellular trafficking, were higher among dorsal/resistant SNpc neurons. SNX8 is a key element of retromer-mediated endosomal protein sorting, also emerging as important cellular machinery in PD. ANXA1 is a key mediator of endoplasmic reticulum (ER) contact sites required for the ER-derived cholesterol transport to endosomes.

**Ion Transport and Homeostasis**

Another shared feature of vulnerable SNpc neurons is their distinctive physiology of autonomous pacemaking activity that mainly relies on Ca\(^{2+}\) channels. However, when these channels are knocked down, a reversal to a more “juvenile” pacemaking activity dependent on Na\(^+\) and hyperpolarization-activated cyclic nucleotide gated (HCN) potassium channels can protect neurons from methylphenyltetrahydropyridine (MPTP)-induced cell death. There are 4 HCN genes in mammals (HCN1–HCN4), each of them showing different biophysical properties. Although HCN4 has been reported to have higher transcript abundance in rodent SNpc neurons, we identified higher HCN1 expression in dorsal/resistant SNpc neurons in humans. HCN1 has been found to be higher with age in the Mitopark mouse, a genetic model of PD with disrupted mitochondrial function, perhaps as a compensatory effect to sustain firing rates and neural function. Our study also identified the expression of SLC4A4 (Na\(^+-\)coupled acid-base transporter), EDN1 (endothelin-1), and PCP4 (Purkinje cell protein 4) as higher in the dorsal SNpc neurons, all genes that maintain Ca\(^{2+}\) homeostasis and thus may be protective. PCP4 (also known as PEP-19) in particular regulates Ca\(^{2+}\) binding to calmodulin, and the levels of transcript and protein of PEP-19 are reduced in striatum of the MPTP mouse model of PD. In addition, we found higher expression of ATP13A4, cation-transporting, P5-type adenosine triphosphatase, among the ventral SNpc neurons. Mutations in this gene have been implicated mainly in the developmental disorders and have been shown to reduce calcium transport in vitro. The related family gene ATP13A2 is actually PARK9 locus, linked to autosomal recessive familial parkinsonism, but we found no differences in its expression between the SNpc tiers.

**Oxidative Stress**

Although oxidative stress processes were not directly overrepresented among the increased genes, genes related to oxidation such as HBB and HBA2 (hemoglobin subunit β/α2) were higher among the dorsal/resistant SNpc neurons. Interestingly, apart from binding to O\(^2-\) and CO\(_2\) in blood, hemoglobin is also present in SNpc DA neurons in mice and humans. It may act as storage for oxygen, providing homeostatic mechanisms for SNpc DA neurons that have an exceptionally high energy requirement. Furthermore, double-labeling immunofluorescence has shown reduced levels of hemoglobin specifically in the neurons with aSyn-immunopositive Lewy bodies. In addition, the expression of MT1G (metallothioneins), a free radical scavenger, was also higher among the dorsal SNpc neurons and has been shown to provide a neuroprotective effect in the rotenone mouse model of PD. The increased SPARC (secreted protein acidic and rich in cysteine) gene in dorsal SNpc neurons was recently linked to PD by in silico study that identified novel proteins involved in Cu and Fe metabolism.

One important goal of our human-specific transcriptomic profiling of selectively vulnerable SNpc populations was to analyze the possible links to known PD genes. Unexpectedly, we did not find any overlap between our DEG and PD familial genes or those genes located within regions of high
LD to SNPs associated with the disease by recent PD meta-
yses. However, we did find 4 increased genes that have
been identified as GWAS hits of modest significance for spor-
dic PD by other smaller studies—3 higher among the dorsal
SNpc neurons: MMRN2 (multimerin 2), ANXA1, and
B2M (beta-2-microglobulin); and 1 higher among the ven-
tral SNpc neurons: AGTR (angiotensin II receptor type 1).

Although RNA-seq is a very powerful technique,
some technical artifacts may be present in the data (eg,
starting concentrations or cDNA quality), and validation
with an independent technique is therefore advisable using
different biological replicates from the same population.
The RT-qPCR validation of our top 10 DEGs largely
confirmed the RNA-seq results, but not always; for exam-
ple, GFAP, being one of the most abundant proteins of
the brain, is likely to be a result of contamination by the
LCM process itself. However, 3 of our DEGs (PCP4, 
BAB3B, and HCN1) showed clearly higher gene expres-
sion in the dorsal versus ventral SNpc neurons. Thus, we
wanted to further examine their protein levels, although it
is important to note that these analyses are entirely obser-
vational rather than validating our RNA-seq results as
such. The relationship between mRNA and protein is
complex, as the steady-state transcript abundances only
partially predict the protein levels, and processes down-
stream of transcription play a strong regulatory role.

Although mass spectrometry–based proteomic analysis
of neurons isolated from postmortem human brain by LCM
is possible, this was beyond the scope of our current
study. Therefore, we examined the protein levels in situ
using immunohistochemistry but also immunoblotting the
2mm punctures from both ventral and dorsal SNpc tiers.
Furthermore, we were interested to see whether we could
observe any changes in the protein levels during the pro-
gression of PD pathology (ie, in different Braak stages).

The PCP4 immunohistochemistry showed extensive
labeling of the processes throughout the SNpc devoid of
any clear cytoplasmic staining of the pigmented DA SNpc
neurons. In the human brain, PCP4 immunoreactivity is
mainly found in the processes of basal ganglia and sub-
stantia nigra, and in the cell bodies of thalamic nuclei,
dentate gyrus, and Purkinje cells. Thus, our RNA-seq
signal with PCP4 likely originates from the processes
that are inevitably included in the LCM procedure, the epitope
of our antibody does not recognize PCP4 in the cell body
(eg, similar to neurofilament staining), or increased
mRNA does not manifest as protein change detectable
with immunohistochemistry. Similarly, the RAB3B pro-
teins are mainly enriched in the presynaptic terminals,
and we detected rather diffuse reactivity throughout the
midbrain. Although the variability between the 2mm
puncture samples was high, PCP4 and HCN1 appeared
to have slightly higher protein levels in the dorsal SNpc,
similar to their gene expression. On the other hand,
RAB3B showed a tendency to higher protein levels in the
ventral SNpc, in contrast to its mRNA, highlighting fur-
ther that mRNA and protein are not necessarily congru-
ent. Finally, we showed that HCN1 protein expression in
the dorsal SNpc increased with progression of PD pathol-
gy, suggesting the possibility of a compensatory effect
similar to that observed in the MitoPark mouse, but this
needs further investigation.

Our comparisons of gene expression variation in
human ventral and dorsal SNpc DA neurons to analo-
gous groups of DA1A/DA-SNC and DA1B/DA-VTA1
characterized in mice identified several genes with
similar orientation in the expression pattern across the
tiers. However, none of these genes was found to be sig-
ificantly differentially expressed in humans. In our
study, only LYPD1 and ANXA1 showed significant dif-
fences in expression, and they were increased in the
opposite direction across the tiers between the 2 species.
Such differences found between humans and mice justify
the importance of a human-focused understanding of the
intrinsic difference between the different populations of
DA neurons.

This study underscores the value of human postmor-
tem transcriptome studies, which to date are hampered by
the scarcity of suitable tissue resources. Future studies
would benefit from higher sample numbers and single-
nuclei sequencing instead of LCM to avoid cell type con-
tamination issues. However, nuclei sequencing is limited
to capturing only a fraction of the cellular mRNA popula-
tion. Human-focused omics atlases such as that described
here provide important references for future research.

Acknowledgment
The work was funded by the Monument Trust Discovery
Award (J-1403) from Parkinson’s UK.

We thank all the individuals who have donated their
brains for research and their families; the Oxford Brain
Bank, supported by the Medical Research Council, Brains
for Dementia Research (Alzheimer Society and Alzheimer
Research UK), Autistica UK, and the National Institute
for Health Research Oxford Biomedical Research Centre;
and Drs P. Dodson and P. Magill from the University of
Oxford for useful discussions.

Author Contributions
Study conception and design: R.W.-M., C.W., L.P.; data
acquisition and analysis: J.M.-S., T.I., L.P.; drafting
the manuscript and figures: J.M.-S., T.I., L.P.
Potential Conflicts of Interest
Nothing to report.

References
1. Parkkinen L, O’Sullivan SS, Collins C, et al. Disentangling the relationship between Lewy bodies and nigral neuronal loss in Parkinson’s disease. J Parkinsons Dis 2011;1:277–286.
2. Damier P, Hirsch EC, Agid Y, Graybiel AM. The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson’s disease. Brain 1999;122(pt 8):1437–1448.
3. Fearnley JM, Lees AJ. Ageing and Parkinson’s disease: substantia nigra regional selectivity. Brain 1991;114(pt 5):2283–2301.
4. Elstner M, Morris CM, Heim K, et al. Expression analysis of dopaminergic neuron diversity by single-cell gene expression profiling. Cell 2016;165:550.
5. Ross GW, Petrovitch H, Abbott RD, et al. Parkinsonian signs and sub- stantia nigra regional selectivity. Brain 1991;114(pt 5):2283–2301.
6. Zheng B, Liao Z, Locascio JJ, et al. PGC-1alpha, a potential therapeutic target for early intervention in Parkinson disease. Neurology 2012;79:2307–2314.
7. Ross GW, Petrovitch H, Abbott RD, et al. Parkinsonian signs and sub- stantia nigra neuron density in decedents elders without PD. Ann Neurol 2004;56:532–539.
8. Zheng B, Liao Z, Locascio JJ, et al. PGC-1alpha, a potential therapeutic target for early intervention in Parkinson’s disease. Sci Transl Med 2010;2:52raz7a.
9. Elstner M, Morris CM, Heim K, et al. Expression analysis of dopaminergic neurons in Parkinson’s disease and aging links transcriptional dysregulation of energy metabolism to cell death. Acta Neuropathol 2011;122:75–86.
10. Simunovic F, Yi M, Wang Y, et al. Gene expression profiling of substantia nigra dopamine neurons: further insights into Parkinson’s disease pathology. Brain 2009;132(pt 7):1795–1809.
11. Grimm J, Mueller A, Hefi F, Rosenthal A. Molecular basis for cate- cholaminergic neuron diversity. Proc Natl Acad Sci U S A 2004;101:13891–13896.
12. Greene JG, Dingledine R, Greenamyre JT. Gene expression profiling of rat midbrain dopamine neurons: implications for selective vulnerability in Parkinsonism. Neurobiol Dis 2005;18:19–31.
13. Chung CY, Seo H, Sonntag KC, et al. Cell type-specific gene expression of midbrain dopaminergic neurons reveals molecules involved in their vulnerability and protection. Hum Mol Genet 2005;14:1709–1725.
14. Poulin JF, Zou J, Drouin-Ouellet J, et al. Defining midbrain dopami-nergic neuron diversity by single-cell gene expression profiling. Cell Rep 2014;9:930–943.
15. La Manno G, Gyllborg D, Codeluppi S, et al. Molecular diversity of midbrain development in mouse, human, and stem cells. Cell 2016;167:566–580.e19.
16. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 2009;10:57–63.
17. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 2016;34:525–527.
18. Risso D, Ngai J, Speed TP, Dudoit S. Normalization of RNA-seq data using factor analysis of control genes or samples. Nat Biotechnol 2014;32:896–902.
19. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
20. Braak H, Del Tredici K, Rub U, et al. Staging of brain pathology related to sporadic Parkinson’s disease. Neurobiol Aging 2003;24:197–211.
21. Miller JA, Ding SL, Sunkin SM, et al. Transcriptional landscape of the prenatal human brain. Nature 2014;508:199–206.
22. Chatr-Aryamontri A, Oughtred R, Boucher L, et al. The BioGRID interaction database: 2017 update. Nucleic Acids Res 2017;45(D1):D369–D379.
23. Honti F, Meader S, Webber C. Unbiased functional clustering of gene variants with a phenotypic-linkage network. PLoS Comput Biol 2014;10.e1003815.
24. Wang X, Park J, Susztak K, et al. Bulk tissue cell type deconvolution with multi-subject single-cell expression reference. Nat Commun 2019;10:380.
25. Damaris S, Sloan SA, Zhang Y, et al. A survey of human brain trans- criptome diversity at the single cell level. Proc Natl Acad Sci U S A 2015;112:7285–7290.
26. Allocco DJ, Kohane IS, Butte AJ. Quantifying the relationship between co-expression, co-regulation and gene function. BMC Bioinformatics 2004;5:18.
27. Marco A, Konikoff C, Karr TL, Kumar S. Relationship between gene co-expression and sharing of transcription factor binding sites in Drosophila melanogaster. Bioinformatics 2009;25:2473–2477.
28. Nalls MA, Pankratz N, Lili CM, et al. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson’s disease. Nat Genet 2014;46:989–993.
29. Shi MM, Shi CH, Xu YM. Rab GTPases: the key players in the molecular pathway of Parkinson’s disease. Front Cell Neurosci 2017;11:81.
30. Chung CY, Koprich JB, Hallett PJ, Isacson O. Functional enhancement and protection of dopaminergic terminals by RAB3B overexpression. Proc Natl Acad Sci U S A 2009;106:22474–22479.
31. Goncalves SA, Macedo D, Raquel H, et al. shRNA-based screen identifies endocytic recycling pathway components that act as genetic modifiers of alpha-synuclein aggregation, secretion and toxicity. PLoS Genet 2016;12:e1005995.
32. Steger M, Dief Z, Dhekn E, et al. Systematic proteomic analysis of LRRK2-mediated Rab GTPase phosphorylation establishes a connection to ciliogenesis. eLife 2017;6.
33. Lai YC, Kondapalli C, Lehnneck R, et al. Phosphoproteomic screening identifies Rab GTPases as novel downstream targets of PINK1. EMBO J 2015;34:2840–2861.
34. Seaman M, Freeman CL. Analysis of the Retromer complex-WASH complex interaction illuminates new avenues to explore in Parkinson disease. Prog Brain Res 2010;183:59.
35. Eden ER, Sanchez-Heras E, Tsapara A, et al. Annexin A1 tethers membrane contact sites that mediate ER to endosome cholesterol transport. Dev Cell 2016;37:473–483.
36. Surmeier DJ, Guzman JN, Sanchez-Padilla J, Goldberg JA. What causes the death of dopaminergic neurons in Parkinson’s disease? Prog Brain Res 2010;183:59–77.
37. Chan CS, Guzman JN, Illic J, et al. ‘Rejuvenation’ protects neurons in mouse models of Parkinson’s disease. Nature 2007;447:1081–1086.
38. Santoro B, Chen S, Luthi A, et al. Molecular and functional heteroge- neity of hyperpolarization-activated pacemaker channels in the mouse CNS. J Neurosci 2000;20:5264–5275.
39. Branch SY, Chen C, Sharma R, et al. Dopaminergic neurons exhibit an age-dependent decline in electrophysiological parameters in the MitoPark mouse model of Parkinson’s disease. J Neurosci 2016;36:4026–4037.
40. Jain A. Endothelin-1: a potential pathological factor in Parkinson’s disease?—From endoplasmic reticulum stress to beyond. J Neurol Sci 2014;344:236–237.
41. Skold K, Svensson M, Nilsson A, et al. Decreased striatal levels of PEP-19 following MPTP lesion in the mouse. J Proteome Res 2006;5:262–269.

42. Gibbons A, Bell L, Udawela M, Dean B. mRNA expression of the P5 ATPase ATP13A4 is increased in Broca’s area from subjects with schizophrenia. World J Biol Psychiatry 2018 Dec 3:1–23.

43. Ramirez A, Heimbach A, Grundemann J, et al. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat Genet 2006;38:1184–1191.

44. Biagioli M, Pinto M, Cesselli D, et al. Unexpected expression of alpha- and beta-globin in mesencephalic dopaminergic neurons and glial cells. Proc Natl Acad Sci U S A 2009;106:15454–15459.

45. Ferrer I, Gomez A, Carmona M, et al. Neuronal hemoglobin is reduced in Alzheimer’s disease, argyrophilic grain disease, Parkinson’s disease, and dementia with Lewy bodies. J Alzheimers Dis 2011;23:537–550.

46. Murakami S, Miyazaki I, Sogawa N, et al. Neuroprotective effects of metallothionein against rotenone-induced myenteric neurodegeneration in parkinsonian mice. Neurotox Res 2014;26:285–298.

47. Kumar A, Gupta S, Sharma P, et al. In silico method for identification of novel copper and iron metabolism proteins in various neurodegenerative disorders. Neurotoxicology 2019;73:50–57.

48. Quadri M, Yang X, Cossu G, et al. An exome study of Parkinson’s disease in Sardinia, a Mediterranean genetic isolate. Neurogenetics 2015;16:55–64.

49. Hu Y, Deng L, Zhang J, et al. A pooling genome-wide association study combining a pathway analysis for typical sporadic Parkinson’s disease in the Han population of Chinese mainland. Mol Neurobiol 2016;53:4302–4318.

50. Mizuta I, Tsunoda T, Satake W, et al. Calbindin 1, fibroblast growth factor 20, and alpha-synuclein in sporadic Parkinson’s disease. Hum Genet 2008;124:89–94.

51. Goldstein B, Speth RC, Trivedi M. Renin-angiotensin system gene expression and neurodegenerative diseases. J Renin Angiotensin Aldosterone Syst 2016;17(3).

52. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 2012;13:227–232.

53. Davis S, Scott C, Ansorge O, Fischer R. Development of a sensitive, scalable method for spatial, cell-type-resolved proteomics of the human brain. J Proteome Res 2019;18:1787–1795.

54. Utal AK, Stopka AL, Roy M, Coleman PD. PEP-19 immunohistochemistry defines the basal ganglia and associated structures in the adult human brain, and is dramatically reduced in Huntington’s disease. Neuroscience 1998;86:1055–1063.