Uncovering sex differences in Parkinson's Disease through metaanalysis of single cell transcriptomic studies

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Abstract

Introduction

Parkinson's disease (PD) is the most common neurodegenerative disease after Alzheimer's, and the most common movement disorder. There are an estimated 6.1 million patients worldwide, with a rapidly increasing incidence and prevalence in recent years (Simon et al., 2020). At the neuropathological level, PD is mainly characterized by progressive degeneration of dopaminergic neurons located in the substantia nigra pars compacta (SNpc) (Lotharius & Brundin, 2002). However, although medulla and midbrain are the most affected areas during the early stages, the involvement spreads in the later stages to cortical areas (Braak et al., 2003; Del Tredici & Braak, 2016). This degeneration leads to the onset of both motor (eg. bradykinesia) and non-motor (eg. depression) symptoms of the disease with no treatment capable of decreasing the progression of PD to date (Bloem et al., 2021; Tolosa et al., 2021).

Despite research efforts, the causes behind PD are not yet fully described. However, researchers have been able to identify molecular hallmarks for this disease, including failure of protein clearance pathways, mitochondrial damage, oxidative stress, excitotoxicity and neuroinflammation (Dong-Chen et al., 2023; Maiti et al., 2017; Panicker et al., 2021). Additionally, it is also reported the existence of risk factors that predispose to the development of sporadic PD (90% of cases). Among the most relevant risk factors are age, the presence of certain genetic variants, exposure to pesticides and sex (Belvisi et al., 2020; Cerri et al., 2019; Vázquez-Vélez & Zoghbi, 2021).

Abundant evidence supports the significant impact of biological sex on various aspects of PD, including incidence, progression, symptoms or response to treatment. The incidence and prevalence of the disease is higher in males, while its age of onset is earlier than in females (DuMont et al., 2023; Picillo et al., 2017). There are also sex differences in the symptomatology, both motor and non-motor. In female PD, tremor, pain, depression and dysphagia are predominant, whereas in male PD, freezing of gait, camptocormia, cognitive impairment and urinary dysfunction are more common (Cerri et al., 2019; Meoni et al., 2020). Likewise, there are sex differences in the pathophysiology of the disease, related to most of the pathological processes of PD, as is the case of the greater activation of microglia

in males or the lower oxidative stress in females (Barko et al., 2022; Cerri et al., 2019; Nicoletti et al., 2023). All these findings support the idea that different molecular mechanisms may be involved in PD depending on the sex of the patient. Some explanations for these events are related to biological, genetic, hormonal or environmental factors, such as the possible anti-inflammatory and neuroprotective effect of estrogens. However, the underlying molecular mechanisms have not yet been fully described (Terrin et al., 2023; Vaidya et al., 2021).

A major difficulty in PD is the great variability of the disease among patients, in terms of symptoms, progression and response to treatment, among others. This fact, coupled with the lack of reliable biomarkers to provide a definitive diagnosis and monitor disease progression, makes clinical management complex (Bloem et al., 2021). This adversity could be overcomed by the application of personalized medicine, where the study of sex differences would have an important impact, since sex-specific prevention and treatment strategies could be implemented. Indeed, in recent years some candidates have been proposed as sex-specific biomarkers (serum urate levels as inverse predictor of PD in male or hypertriglyceridemia as a predictor of cognitive impairment in women, among others), although the data are still far from conclusive (Ascherio et al., 2009; Luca et al., 2022; Nicoletti et al., 2023). To address this problem, high-throughput technologies, such as single cell RNA-seq (scRNA-seq), allow us to assess these differences in a more global and comprehensive manner. In addition, by integrating the numerous omics studies available in public repositories, the difficulty of inter-patient variability can also be overcome. With this in mind, we now present an integration of 6 single-cell transcriptomic studies performed on postmortem brain tissue from PD patients, with 2 cell-type-specific meta-analyses of different brain regions.

To integrate data, we use the meta-analysis strategy, which provides greater robustness to the results obtained and avoids the variability associated with each individual study. Our results show sex differences in gene expression, cell-cell communication and pathway activation in all major brain cell types, highlighting the presence of greater neuroinflammation in men and greater neurodegeneration in the SNpc in men, with the latter appearing to be inverted between sexes when observed in the cortical zone. Finally, we have made all the results available in a publicly accessible webtool, in order to allow the exploration of results to other researchers and to broaden the molecular-level understanding of PD sex differences.

Materials And Methods

The bioinformatics processing and analysis was carried out using the R programming language (R Core Team, 2021) (version 4.1.2). The packages and versions used are listed in Table S1.

1. Study selection

A systematic review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria (Liberati et al., 2009; Page et al., 2021), whose main objectives are standardization and reproducibility. First, we searched public repositories such as GEO (Gene Expression Omnibus) (Edgar et al., 2002), ArrayExpress

(Athar et al., 2019), BioStudies (Sarkans et al., 2018), ENCODE (Y. Luo et al., 2020), UCSC Cell Browser (Kent et al., 2002), Human Cell Atlas (Regev et al., 2017), and Single Cell Expression Atlas (Moreno et al., 2022) using the keywords: "Parkinson's", "Parkinson's disease" or "PD". To identify suitable datasets, we set the following inclusion criteria: i) organism: *Homo sapiens* and, ii) study type: single-cell or single nuclei RNA sequencing (scRNA-seq and snRNA-seq). Among the selected studies, we discarded those that met any of the established exclusion criteria: i) experimental design not involving PD patients versus controls, ii) not focused on PD, iii) lack of information on patient sex, iv) lack of representation of both sexes in all conditions, and v) samples not from post-mortem brain tissue (fetal, organoid, etc.). Finally, the raw count data and associated metadata of the selected studies were downloaded either manually or using the R package GEOquery (Davis & Meltzer, 2007).

2. Individual analysis

All selected studies were individually analyzed following three main steps: snRNA-seq data processing, cell type annotation, and differential expression analysis. We performed these analyses based on the guidelines proposed by Amezquita et al., which are based on the use of packages from the Bioconductor repository (Amezquita et al., 2020).

a. snRNA-seq data processing

All downloaded data from the different studies (count matrix, cell data, patient metadata, etc.) were stored in a single SingleCellExperiment (SCE) using the SingleCellExperiment R package (Amezquita et al., 2020), or the read10xCounts function of the DropletUtils R package (Lun et al., 2019), depending on the available data format. Afterwards, we performed a homogenization of the nomenclature used for the genes to enable integration between studies.

We then perform quality control to discard droplet counts not derived from a single viable cell. To detect the empty droplets, we used the original paper thresholds for the number of Unique Molecular Identifiers (UMIs) and the number of detected genes. We also discarded those droplets with a percentage of mitochondrial genes higher than 5%. Genes with less than 5 counts were also removed. Finally, we considered as doublets those droplets identified by the scDblFinder R package, as well as those with a library size larger than 4 MADs (Mean Absolute Deviation), since such abundant gene expression is not expected in brain cells. Normalization was performed using the scran (Lun et al., 2016) and scater R packages.

We selected the top 25% of the most variable genes and performed a principal component analysis (PCA) for dimensionality reduction, using scran and scater packages respectively. We next used the PCAtools R package (Blighe & Lun, 2021) to select the number of principal components (PCs) that summarize the largest amount of variance. These selected PCs were used for the T-distributed Stochastic Neighbour Embedding (tSNE) and the Uniform Manifold Approximation and Projection (UMAP) constructed for visualization purposes. Finally, we performed cell clustering using the scran package, applying a combination of the k-means and Shared Nearest Neighbours algorithms. The number of

centroids and neighbours were adapted to the number of cells in each study, in order to maintain a similar resolution between them.

b. Cell type annotation

Major cell types were annotated combining unsupervised strategies to increase robustness and avoid individual analysis bias. We integrated annotation approaches based on full transcriptomic references and marker gene expression profiles.

For the reference expression profiles-based approach we use the singleR (Aran et al., 2019) and CHETAH (de Kanter et al., 2019) packages, using in both cases as reference dataset a subset of the Allen Brain Map atlas (Bakken et al., 2021), adapted to the recommendations of each software. For the marker gene approach, we used the SCINA R package (Z. Zhang, 2019), using the reference markers present in the CellMarker database (X. Zhang et al., 2019), and BRETIGEA R package (McKenzie et al., 2021), using the package's own marker genes. Subsequently, to obtain the consensus annotation, we assigned each cell as the most frequent annotation among all the methods, being 2 the minimum number of algorithms that must coincide in the same annotation. Those cells with indeterminate annotation or not annotated as the majority cell type of the cluster were discarded to avoid possible biases in subsequent analyses.

We annotated astrocytes, neurons, microglia and oligodendrocytes in the different studies, as these are the 4 major cell types in brain tissue. Clusters that were not correctly annotated were discarded for the visualization of results and they were not used in the downstream analyses.

Finally, the expression of marker genes tested in the original studies were evaluated using the scater's plotExpression function to check that the annotations were performed correctly.

c. Differential expression analysis

Differential expression analyses (DEA) were performed using the MAST R package (McDavid et al., 2021). First we built a hurdle model with the zlm function, giving as the main variable the combination of condition and sex. We also used the sample that each nucleus came from to reduce intra-patient bias. With this model, we used functions lrTest to obtain the p-values and getLogFC to obtain the logarithm of fold change (logFC) and the standard deviation (SD) of the genes. To obtain the sex-based differentially expressed genes (DEGs), we applied the following contrast:

(PD_female − Control_female) − (PD_male − Control_male)

This contrast reveals the sex differential impact of disease, identifying those genes whose expression contributes to the pathophysiology of the PD in a manner that differs depending on the sex of the patient, taking into account the basal differences at the physiological level that may exist between healthy males and females.

In this context, a positive logFC in this contrast could mean that, during PD progression, the gene: i) is upregulated in females and downregulated in males; ii) is upregulated in both sexes, but the increase in expression is higher in females than in males; iii) is downregulated

in both sexes and the decrease in expression is lower in females. For simplicity, we refer to these genes as increased in females. Otherwise, the opposite is interpreted for genes associated with a negative logFC, which we designate as increased in males.

d. Signaling pathway activation analysis

The hipathia R package (Hidalgo et al., 2016) was used to compute the activation values of the KEGG signaling pathways from the normalized gene expression matrices of the individual single cell datasets. This software divides each pathway into distinct sub-pathways associated with a particular end node or effector of the pathway, obtaining an activation value for each one. Thus, with this step we obtain the activation levels of each sub-pathway in each cell of the dataset. Subsequently, we perform a differential activation analysis (DAA) using MAST and the sex-differential double contrast as described in the previous section. We considered differentially activated sub-pathways (DA-subPs) those with a FDR lower than 0.05.

3. Meta-analysis

DEA and DAA results were integrated into 2 meta-analyses, one including datasets of the cortical region and other of the nigrostriatal pathway. The meta-analyses were performed using the R package metafor (Viechtbauer, 2010), using the DerSimonian and Laird random effects model, which accounts for the heterogeneity of each individual study, giving greater weight to those studies with lower dispersion. These analyses calculate for each gene its p-value, logFC and SD among other statistics. The p-value was corrected using the Benjamini & Hochberg (BH) algorithm, and those genes with a p-adjusted value < 0.05 and a logFC > 0.5 were considered significant DEGs. In the case of pathways, they were considered DA-subPs when the p-adjusted p-value was lower than 0.05. Finally, we applied diagnostic tests on the obtained results to avoid possible bias effects, including graphical representation of DEGs with funnel and forest plots, and influence and sensitivity analyses (leave-one-out cross-validation).

4. Functional enrichment

We performed a biological characterization for each cell type in both sexes from its increased DEGs. The weight01 algorithm (Alexa et al., 2006) from the topGO R package (Alexa & Rahnenfuhrer, 2023) was used for the Biological Process (BP) GO ontology with a minimum node size of 15 genes.

The significant GO terms obtained (p-value < 0.05) were further processed with the R package simplifyEnrichment, to obtain clusters of similar GO terms, which summarize and facilitate interpretation. For better visualization, these clusters are represented in a similarity heatmap with their associated word clouds. Also, to facilitate the visualization and understanding of the data, we selected the ancestral GO term that best covered the information of each cluster. To select it, we took into account the frequency of appearance of the ancestor within the cluster and its probability of appearance in the GO database, an index obtained using the GOSemSim package.

Protein-protein interaction (PPI) networks were created with the STRINGdb R package, using a threshold score of 400. These networks were then analyzed and modified through the STRING web tool (Snel et al., 2000; Szklarczyk et al., 2020).

5. Cell-Cell Communication analysis

Cellular communication between the different cell types was analyzed using the iTalk R package (Y. Wang et al., 2019). We used Omnipath intercell resource, from the OmnipathR R package (Türei et al., 2021), as the database for ligand-receptor interactions, since it integrates most of the available resources of ligand-receptor databases, and also has a curated specific category for intercellular communication analysis. We also used this resource to tag each sender and receiver protein with its feature and functional annotations.

For the analysis, we used DEGs obtained from the meta-analysis as input, assuming as sex-specific interaction gains those whose ligand and receptor are both significant in the meta-analysis for that sex. We used the product of the logFC from the sender and receiver as a measure of intensity in the gain of interaction. We also used the logFC for the representation of the results in circus plots.

6. Web platform

We developed a user-friendly online resource to easily explore all the results obtained from the different analyses conducted in this work. The web app (https://bioinfo.cipf.es/cbl-atlas-pd/) was developed under the structure of the R shiny package (Chang W, 2024) and is compartmentalized into the different steps performed in the analysis.

Results

Study selection and individual analysis

The systematic review identified 59 unduplicated studies (Figure 1A). Of these, 7 studies were selected after applying the established inclusion and exclusion criteria (further details in material and methods section). After quality control analyses, one of them (GSE184950 (Q. Wang et al., 2024)) was discarded because the majority of the cells had too high expression of mitochondrial genes. Therefore, 6 studies (Table 1) with a total of 110 samples, were included in the final analysis. Sample distribution by sex and condition is reported in Figure 1B. Of these 6 studies, 4 were from the nigrostriatal pathway (3 from the substantia nigra and 1 from the striatum) (Kamath et al., 2021; A. J. Lee et al., 2022; Smajić et al., 2021; Xu et al., 2023) and 2 from anterior cortical areas (1 from the anterior cingulate cortex and 1 from the prefrontal cortex) (Feleke et al., 2021; Zhu et al., 2022).

After performing the individual dataset processing, we identified neurons, astrocytes, microglia and oligodendrocytes in each study. The distribution of cell types per study is illustrated in Figure 1C. GSE157783 study cell types were represented in a UMAP embedding (Figure 1D) and the expression of marker genes in each cluster was verified

(Figure 1E). Results obtained in the rest of the datasets are similar and can be found in Figure S1. Finally, differential expression analyses identified DEGs in both sexes for all cell types in all studies, except for the case of microglia in study GSE148434 (Figure 1F).

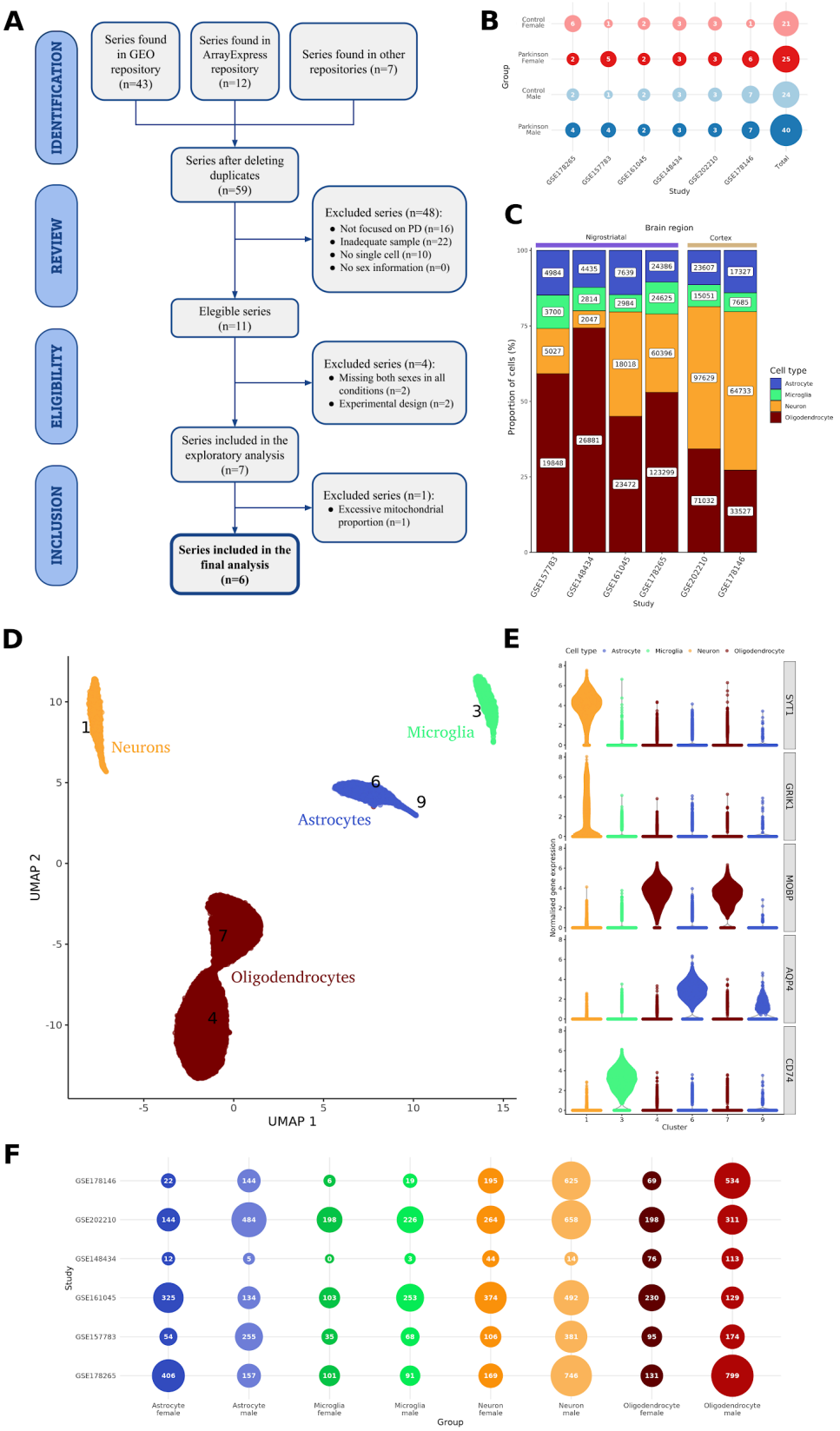

Figure 1 - Systematic review and individual analyses results overview. A- PRISMA diagram with the description of the different steps performed for the systematic review. B- Number of patients from each selected study, grouped by sex-disease condition. C- Barplot showing the proportion and total number of each cell type identified by study. D- UMAP embedding of GSE157783 study, colored by cell type. Numbers correspond to clusters. E- Violin plot representation of the expression of cell type markers for each of the clusters of the GSE157783 study, colored by cell type. F- Number of significant DEGs in each individual differential expression analysis, classified by cell type and sex in which the gene is increased. *DEGs: differentially expressed genes, PD: Parkinson's disease,* PRISMA*: Preferred Reporting Items for Systematic Reviews and Meta-Analyses, UMAP: Uniform Manifold Approximation and Projection.*

Table 1 - Description of the selected datasets. * Control female : case female : control male : case male. SNpc: Substantia nigra pars compacta; ACC: Anterior cingulate cortex; PC: Prefrontal cortex.

Dataset	Sequencing method	Sequencing platform	Brain region	Tissue	Patients*	Cell number	Gene number	Genome aligned	Ref
GSE178146	10x Genomics	Illumina HiSeq 4000	Cortex	ACC	1 : 7 : 6 : 7	156.577	25907	GRCh38	
GSE202210	10x Genomics	Illumina NovaSeq 6000	Cortex	PC	3 : 3 : 3 : 3	232.155	27253	GRCh38	
GSE178265	10x Genomics	Illumina NovaSeq 6000	Nigrostriatal	SNpc	6 : 2 : 2 : 4	253.029	24020	GRCh37	
GSE157783	10x Genomics	Illumina NovaSeq 6000	Nigrostriatal	Midbrain	1 : 1 : 5 : 4	39.110	23383	GRCh38	
GSE161045	10x Genomics	Illumina NovaSeq 6000	Nigrostriatal	Putamen	2 : 2 : 2 : 2	57.228	32101	GRCh38	
GSE148434	10x Genomics	Illumina NovaSeq 6000	Nigrostriatal	SNpc	3 : 3 : 3 : 3	41.455	20779	GRCh37	

Gene expression meta-analysis

We performed two cell-type-specific meta-analyses of gene expression, one from the four studies of the nigrostriatal pathway (NS) and the other from the two studies of cortical (C) regions. We categorized these DEGs as increased in females or males depending on whether the logFC obtained from the double contrast was positive or negative, respectively.

Figure 2A shows the results obtained from the NS meta-analysis. We found a total of 554 DEGs across all cell types (89 in astrocytes, 83 in microglia, 252 in neurons and 130 in oligodendrocytes), distributed between the two sexes. Regarding cortical meta-analysis, it revealed 1219 DEGs (127 DEGs in astrocytes, 73 in microglia, 920 in neurons and 99 in oligodendrocytes) (Figure 2C). In both meta-analyses, neurons presented the highest number of DEGs, which may indicate a greater impact of sex in the disease on this cell type. To further investigate this, we represented these results in a volcano plot for each brain region (Figure 2B for NS and Figure 2D for C), labeling those with greater significance and variation between sexes. All DEGs obtained in all cell types for both meta-analysis can be found in Table S2. The online resource (https://bioinfo.cipf.es/cbl-atlas-pd/) also allows

further exploration of these results, along with results of sex-stratified analyses (DEA in each sex separately).

Additionally, we individually explore the forest plots of some DEGs with biological relevance in the NS meta-analyses, to check that there were no studies that significantly influenced the overall result of the meta-analysis (Figure 2E). We found of special interest DEGs GRIA2 in astrocytes, an AMPA-type glutamate receptor that might act as a glutamate sensor in astrocytes; PIBF1 in microglia, a gene related with progesterone and with immunomodulatory functions; and VPS35 in neurons, related with the retromer complex and described as involved in Parkinson's disease.

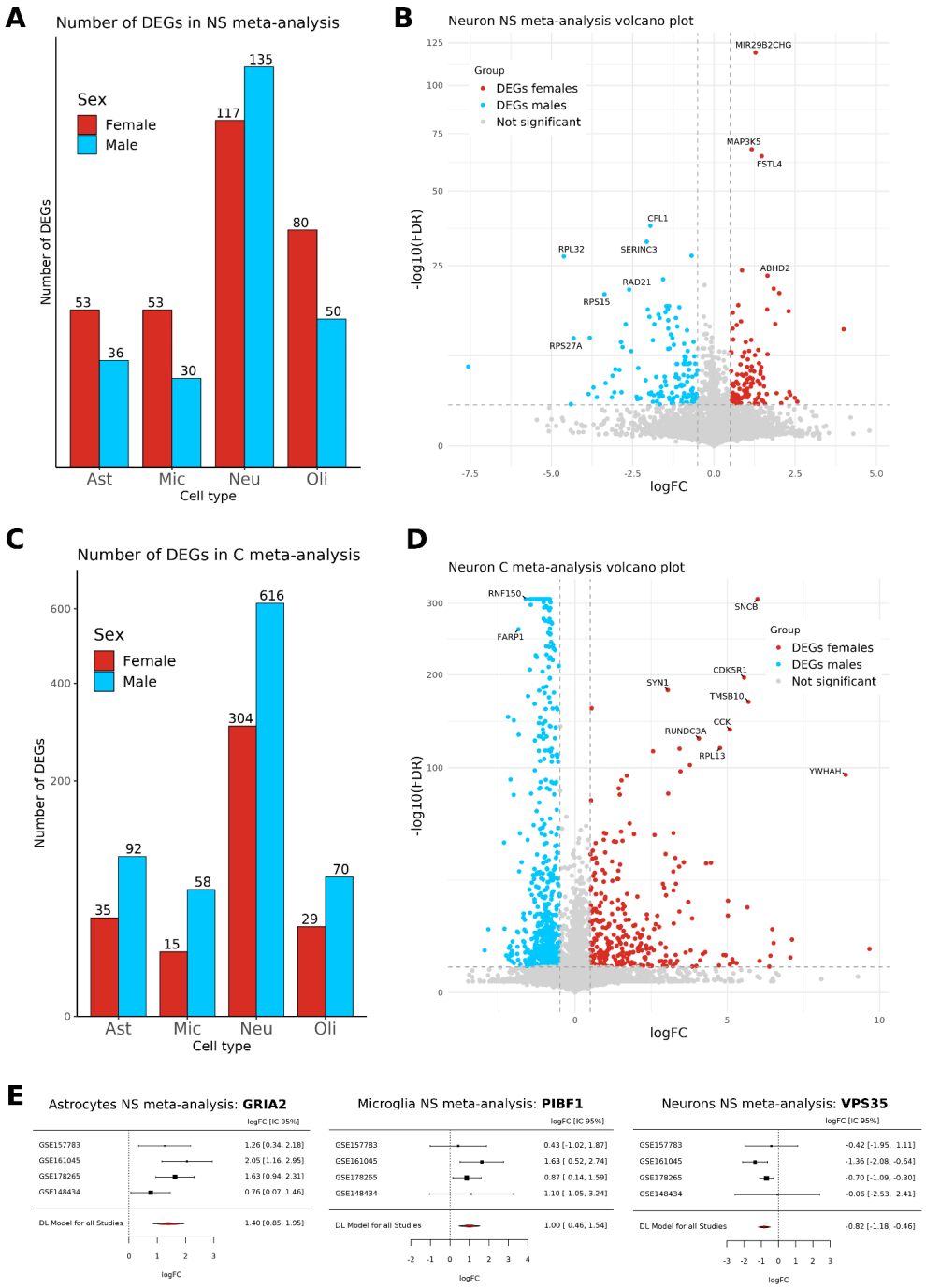

Figure 2 - Meta-analyses results of nigrostriatal pathway region. A,C- Number of DEGs obtained in the NS (**A**) and C (**C**) meta-analyses for each cell type, divided by sex. **B,D-** Volcano plot representation of the results obtained in the NS (**B**) and C (**D**) meta-analysis of neurons. The x-axis represents the logFC of the comparative and the y-axis the -log10 of the adjusted p-value. Those identified as DEGs (FDR < 0.05 & abs(logFC) > 0.5) are colored by sex. **E-** Forest plot of GRIA2 (left), PIBF1 (middle) and VPS35 (right) genes in NS meta-analysis for different cell-types. The forest plots show the logFC and confidence intervals for each individual study and the DL model for all studies (lower red diamond). Ast: Astrocytes, Mic: Microglia, Neu: Neurons, Oli: Oligodendrocytes, NS: Nigrostriatal, C: Cortical, DEGs: differentially expressed genes, FDR: false discovery rate, IC: confidence interval, DL: DerSimonian and Laird.

Biological characterization of gene meta-analysis

The overrepresentation analysis showed significantly overrepresented GO biological process terms in all groups of DEGs from the meta-analysis of both brain regions. The number of significant GO terms for the NS and C regions is shown in Figure 3A and S2A, respectively. In the NS, we observed that the number of terms in males is higher than in females, despite the fact that male gene sets are, in most cases, smaller than those of females (Figure 2A). Concerning C region, the opposite occurs with non-neuronal cell types, with more GO terms obtained for females, despite the starting gene sets being smaller (Figure 2C).

For interpretability purposes, these GO terms sets were grouped based on their similarity, obtaining clusters of terms related to similar functions. Then, each cluster was also associated with its most representative GO term, which was obtained by taking into account the frequency of appearance of the ancestor within the cluster and the “rarity” of the GO term (see Methods Section 4). We represented all this information in Figure 3C and Figure S2B for all cell types and sexes in NS and C regions, respectively. All significant GO terms categorized by cluster can be found in Table S3 for the NS and Table S4 for the C region. In addition, the web resource provides more information on this analysis and allows the realization of customized plots on the biological processes of interest (https://bioinfo.cipf.es/cbl-atlas-pd/).

Focusing on the NS, Figure 3B shows a graphical summary of the main results obtained for each of the cell types analyzed. In general terms, processes increased in females are related to the synapses and their maintenance (i.e., axon extension (GO:0045773) in neurons, neurotransmitter reuptake (GO:0098810) in astrocytes), methylation processes (i.e., GO:0016571 in microglia), and some regenerative processes mediated by glia (i.e., healing of damage (GO:0042060) in microglia, organization of vesicles (GO:0051650) in oligodendrocytes). Concerning males, processes point to an increased immune response (i.e., myeloid leukocyte cytokine production (GO:0061082) in microglia), neuronal death (i.e., positive regulation of intrinsic apoptotic signaling pathway (GO:2001244) in neurons) and ribosome-related processes (i.e., cytoplasmic translation (GO:0002181) in neurons and oligodendrocytes).

Additionally, we performed term clustering of the overrepresented GO terms from different cell types to identify both common and cell-type specific functional modules. The term

clustering in females, including neurons, microglia and astrocytes, showed many of the processes described above, highlighting cluster 1 (axon extension) and cluster 2 (immune processes), predominantly observed in microglia and neurons, and cluster 5 (neurotransmitter reuptake), mainly from astrocytes. In contrast, the term clustering in males did not reveal any cell-type specific clusters. However, it is notable that microglia, neurons and oligodendrocytes shared a greater number of clusters than was observed in females (Figure 3E), emphasizing cluster 1 and cluster 3, both related to the immune response.

We explore in a more detailed way the functions obtained in male astrocytes, since they did not cluster as well as the rest of cell types. Among the top overrepresented GO terms (Figure 3F), we detected terms related to metal homeostasis and lipid metabolism, which can be related with the ferroptosis process, widely described as involved in PD. When we further explored the DEGs related to these processes, we found ceruloplasmin (CP), both chains of Ferritin (FTH1 and FTL) and metallothionein MT3, for the metal homeostasis, and FABP5 or APOD for the lipid metabolism, among others.

In addition, we also build PPI networks in each gene set described, obtaining in most cases significantly interconnected networks. Here we highlight the DEGs PPI network of male neurons (Figure 3G), as it is the largest gene set with the highest number of associated GO terms. We observed that most proteins formed a functional module, highlighting the group of highly interconnected ribosomal proteins, ubiquitination enzymes (UBB, UBC, UBE2N, among others) and some proteins related to secretion, including SORL1 and VPS35.

Finally, in the cell communication analysis we observed that the differential interactions based on sex were mainly between oligodendrocytes and neurons. Among the interactions with the highest logFC in ligand and receptor, we highlight APOE-SORL1 in females and RET-MAPK8 in males between oligodendrocytes and neurons, and SEMA5A-PLXNA2 in males between neurons (Figure 3H).

As for the meta-analysis of the C region, the processes obtained are not as comprehensible as in the case of the nigrostriatal pathway between cell types. In female neurons, despite finding processes related to synapses, we also identify processes related to cell death. To fully characterize this, we explore its PPI network, and we found processes related to ribosomes, neuron system development, gene expression inhibition, PD and apoptosis, among others (Figure S2C). Concerning other cell types, we can highlight anti-inflammatory and myelinating processes carried out by the microglia (response to interleukin-4 (GO:0070670)) and oligodendrocytes (central nervous system myelination (GO:0022010)) respectively.

Regarding males, there are patterns of neuronal survival and synapse strengthening, along with adhesion processes, observed both in individual enrichments (i.e. neurotransmitter reuptake (GO:0098810) and axon guidance (GO:0007411) in astrocytes, neuron projection morphogenesis (GO:0048812) and glutamatergic synaptic transmission (GO:0035249) in neurons, etc.) and in neuron-astrocyte GO term clustering (Figure S2D). Regarding male microglia, we observed processes related to phagocytosis and chemotaxis (positive regulation of phagocytosis (GO:0050766)), highlighting the increase of the CX3CR1 receptor.

Lastly, in the cell-cell communication analysis (Figure S2E), we notice a more important involvement of astrocytes compared with the NS region. Among the interactions with the highest rate of change in females are LIFR-IL6ST between astrocytes and microglia or NRG2-ERBB4 between neurons. Meanwhile, in males, we highlight APLP1-PRNP interaction between neurons and oligodendrocytes, CTTN-ACTB between neurons and microglia or CALM3-CAMK2G between neurons.

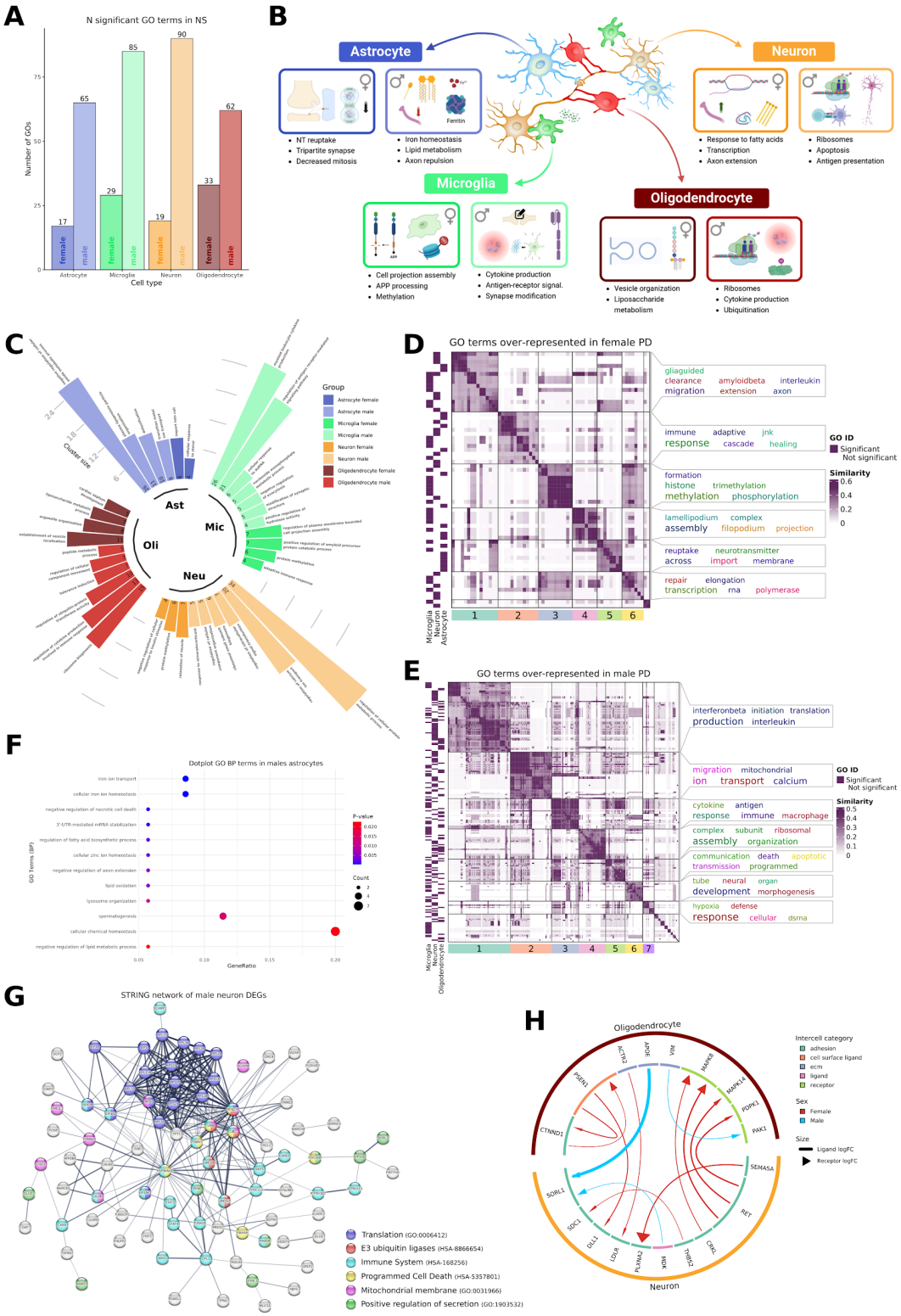

Figure 3 - Biological characterization of the results obtained in the meta-analysis of the nigrostriatal pathway. A- Number of significant GO terms for each of the cell types separated by sex. **B-** Graphical overview of the biological results obtained in each of the cell types for each sex. **C-** Circular barplot representing the size of the GO term clusters obtained for each of the cell types and sexes. Above the bars, the name of the GO term that best summarizes each of the identified clusters. **D,E-** Heatmap representation of the similarity matrix obtained from GO biological processes enriched in neurons, astrocytes and microglia in females (D) and neurons, microglia and oligodendrocytes in males (E). Bottom divisions delimit terms grouped in clusters, and on the right side, the word cloud with the most representative words of the cluster. Left margin represents from which cell type each of the GO terms comes from (color indicating presence and blank absence). **F-** Dotplot of top 12 GO biological processes overrepresented in male astrocytes, ordered by p-value. **G-** PPI network of the males DEGs obtained in the meta-analysis of neurons. The network was obtained with mean interaction confidence (0.400). Each node is colored by its associated enriched functional term. **H-** Circos plot with the cell-cell interactions classified by sender and receiver cell type and by function of each gene in the intercellular interaction. The thicknesses of the body and tips of the arrows correspond to the logFC magnitude of the ligand and receptor, respectively. PPI: Protein-protein interaction, GO: Gene Ontology, BP: Biological Processes, NT: Neurotransmitter, Ast: Astrocytes, Mic: Microglia, Neu: Neurons, Oli: Oligodendrocytes, NS: Nigrostriatal pathway, DEGs: differentially expressed genes, logFC: logarithm of fold change.

Pathway activation meta-analysis

To further describe the biological processes involved, we analyzed the differential sex-specific activation patterns of the KEGG signaling pathways. This analysis divides each pathway into distinct sub-pathways associated with a particular effector protein, obtaining an activation value for each one. We compared these activations using the same double contrast described for the gene meta-analysis, thus obtaining sex-dependent differentially activated sub-pathways (DA-subPs). With this, we identified sex-dependent activation patterns in all cell types. For synthesis purposes, all the DA-subPs were aggregated by pathway and, these pathways, were categorized based on its associated biological function (Figure 4A, Figure S3A).

With regard to the NS, the results obtained by sex are consistent with those observed in the previous analysis, highlighting again that neurons are the ones with the highest number of DAsubPs, regardless of sex. In a more detailed way, in females, there is a greater number of processes related to synapses and those related to cell death and infectious diseases are more common in males. The former ones may indicate a greater immune response, especially in microglia, since its activation in neurons and oligodendrocytes is due to the increase of ribosomal genes, as they also appear in these pathways. It is also notable the increased presence of adhesion pathways in male oligodendrocytes.

To obtain a complete overview of each process and combine these results with the gene meta-analyses, we plotted the significantly activated subpathways with more biological interest in this context together with the logFCs from the previous gene meta-analysis. In

general terms, pathways with increased activation in females have mostly increased genes in females (positive logFC) and vice versa (Figure 4B-C).

In glutamatergic and GABAergic synapses (Figure 4B), activated in female neurons, subpathways involved are related to the proper functions of these synapses, either favoring hyperpolarization or reducing the excitability of the postsynaptic neuron, respectively. In the case of astrocytes (Figure 4C), the effectors activated in males in the *Axon guidance* (top) pathway lead mainly to axonal repulsion, and in the *PPAR pathway* (bottom), activated in females, is mainly related to fatty acid metabolism, as the activated effector is the Long-Chain Fatty Acid Transport Protein 4 (SLC27A4). The rest of the pathways, together with the significant effector circuits by cell type, can be viewed in the associated web resource (https://bioinfo.cipf.es/cbl-atlas-pd/).

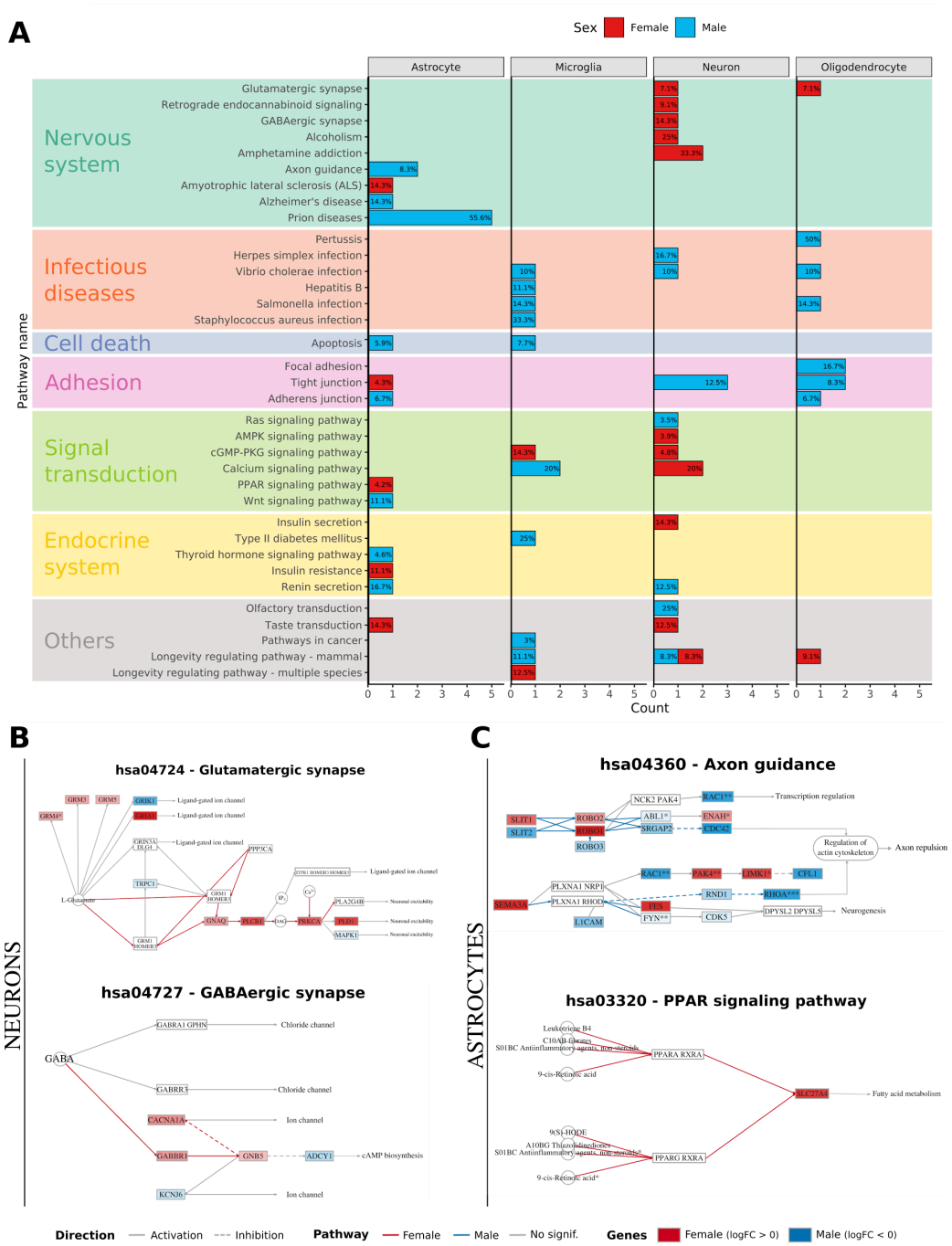

Figure 4 - Results of pathway meta-analysis of NS. A- Differentially activated signaling pathways in the meta-analysis of each cell type, coloured by sex. X-axis indicates the number of significant subpathways and the label inside the bar indicates the percentage over the total number of subpathways in the corresponding pathway. B & C- Graphs representing differentially activated signaling pathways in neurons (B) and astrocytes (C). Nodes represent genes and edges and pathways. Coloured edges correspond to significant differentially activated subpathways. The color intensity of the genes is proportional to their FDR in the gene meta-analysis (the lower the FDR, the higher the intensity). To improve visualization, we discarded non-significant subpathways that are not involved in the significant ones.

In the C area, most significant subpathways appeared more activated in males, except for the microglia. Among these results, the numerous synapse pathways activated in male neurons or the presence of the apoptosis pathway in female neurons stand out (Figure S3A). Regarding the apoptosis pathway, there is only one significant subpathway differentially activated, (TUBA1B effector), however when visualizing this pathway we see that many of the effectors show a tendency to be increased in females (Figure S3B).

Comparison between nigrostriatal and cortex meta-analyses

We next compared the meta-analyses results between brain regions in order to characterize sex differential expression profiles in brain regions involved in different stages of the disease. When we intersected the genetic signatures of each sex in each cell region for all cell types analyzed, neurons stood out as having a reversed pattern between sexes. That is, neurons shared more genes with opposite sex than within the same sex (i.e., 28 common DEGs increased in NS male and in C female, while only 1 DEG is common between NS female and C female) (Figure 5A). In an exploratory analysis of protein-protein interaction in the genes of these intersections, we observed that functional modules may exist in both cases. The common DEGs between NS males and C females are mainly related to ribosomes, apoptosis, and the immune system, whereas the common DEGs between NS females and C males that are connected are more related to the synapse. Both networks obtained significantly more iterations than expected (PPI enrichment p-value: NS-female & C-male: 0.0005, NS-female & C-male: 5.49e-05).

To further characterize this inverse pattern between these brain regions in neurons, we clusterized the significant overrepresented processes of opposite sexes. NS male with C female (Figure 5B) presented a combination of the terms from both brain regions, as any cluster was identified region-specific. The two largest clusters are related to translation and post-translational modifications of proteins and to the assembly and organization of cellular complexes, respectively, possibly related to ribosomal processes. Cluster 3 and 4 are also remarkable, with terms related to calcium signaling and to the intrinsic pathway of apoptosis, respectively. On the contrary, clustering from C males and NS females is related with synapse and neuron development, although the terms of the clusters are not so evenly distributed concerning brain regions (Figure S4A).

We also performed these analyses with microglia to test the nature of the immune response. In this case, the similar patterns were found within the same sex (Figure 5C). In both brain regions we observed processes related to a more exacerbated immune response in males. In cluster 1 the most representative term is regulation of antigen receptor-mediated signaling pathway (GO:0050851), and cluster 2 refers to a cellular response (GO:0071359). In the females clustering, there is a cluster referring to immune homeostasis and methylation, reinforcing the hypothesis of a similar immune response for both brain regions. (Figure S4B).

In the signaling pathway analysis we also observed common dysregulated pathways between both brain regions in astrocytes, neurons and oligodendrocytes (Figure 5D). In most cases, their activation occurs in opposite sexes, highlighting the glutamatergic and GABAergic synapse pathways in neurons, increased in females in NS and in males in C. When the latter pathway is represented in more detail, a practically opposite pattern is observed both at the level of subpathways and logFC of genes involved (Figure 5E).

Finally, in the analysis of cell-cell communication, a greater number of interactions were identified in the C than in the NS, probably due to the greater number of DEGs obtained in this region for all cell types (Figure 2A,C). However, while there are more interactions in females in the NS (13 females vs 3 males), the opposite is true for cortex (167 males vs 57 females) (Figure S4C). No common interactions were detected between both brain regions (Table S5).

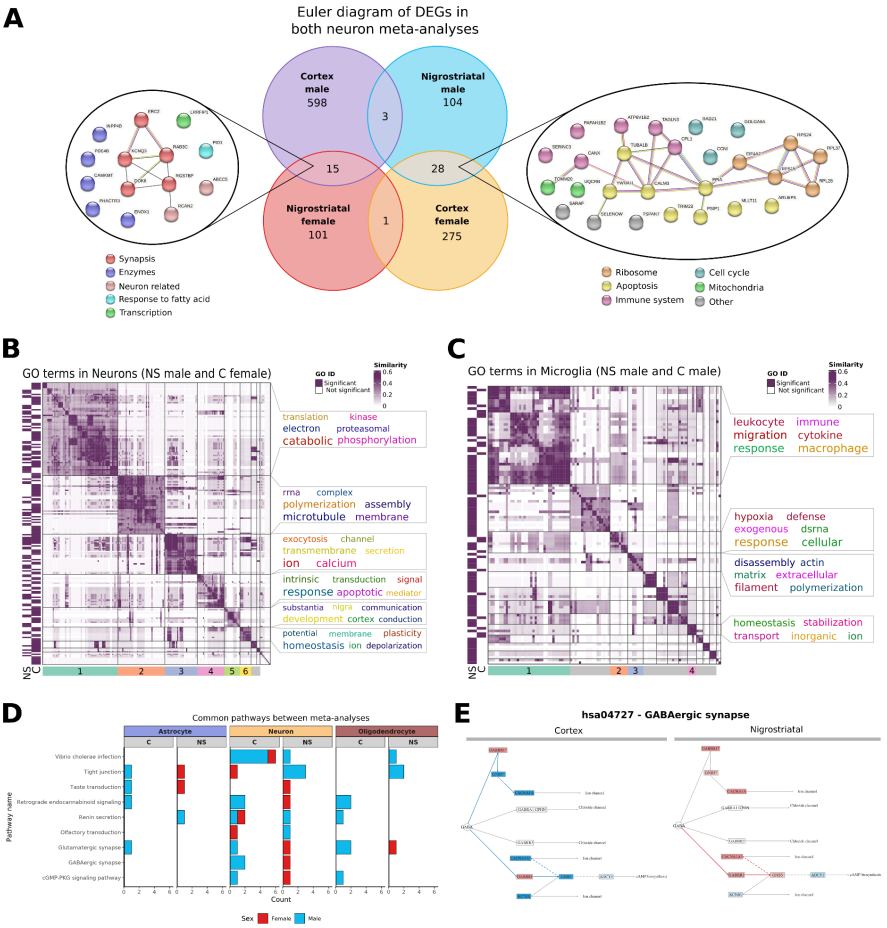

Figure 5 - Comparison between the results obtained in the meta-analysis of the NS and C circuits. A- Euler diagram with the neuron DEGs obtained in the meta-analysis of the nigrostriatal circuit and the cortex. Networks were obtained for intersections between opposite patterns, with an interaction confidence score threshold of 0.150. Through a literature search, we identify functions related to the genes and color them accordingly. B & C - Heatmap representation of the similarity matrix obtained from GO biological processes enriched in NS male and C female meta-analyses in neurons (B) and NS male and C male meta-analyses in microglia (C). Clusters are displayed at the bottom, and a wordcloud featuring the most significant terms within each cluster on the right side. Brain region to which each process belongs is illustrated on the left. D - Significant activated pathways in opposite sexes in the same cell type between NS and C meta-analyses. E - Graph of the GABAergic synapse signaling pathway (*hsa04727*) in both meta-analyses. GO: Gene Ontology, C: Cortical area, NS: Nigrostriatal pathway, DEGs: differentially expressed genes.

Discussion

With our meta-analyses of the NS and C regions we evidence that there are differences between males and females at single cell transcriptome resolution level, with novel processes that deepen the understanding of sex differences in PD. Furthermore, our results are in accordance with the currently reported evidence about these differences (Cerri et al., 2019; DuMont et al., 2023; Nicoletti et al., 2023), including at the transcriptomic level (López-Cerdán et al., 2022). Our discussion will particularly emphasize the results obtained from the NS meta-analysis, since there are more studies from this region that support these results, and therefore, they are more robust than those obtained in the C meta-analysis.

Astrocytes can conduct both protective and detrimental functions in PD, adopting reactive states in response to the pathology stimuli (Liu et al., 2023; Patani et al., 2023). Our results suggest that some of these protective mechanisms are more prominent depending on the sex of the patient.

Male astrocytes dispose of functions related to iron homeostasis and lipid metabolism that may be related to ferroptosis, which is associated with neuronal death in neurodegenerative diseases (Zecca et al., 2004). However, the genes involved are related to protective processes against oxidative stress-induced ferroptosis. These genes are the ferritin (FTH1 and FTL) and ceruloplasmin (CP) genes, which are responsible for oxidizing and storing iron in a non-toxic form respectively, and which may be a neuroprotective mechanism for astrocytes in PD (N. Zhang et al., 2022). Indeed, CP is essential in astrocytes as it is its only ferroxidase and its absence causes remarkable morphological abnormalities, oxidative stress, specially in the basal ganglia, among other tissues. This protein, together with FLT, have been related with syndromes of neurodegeneration with brain iron accumulation (Hinarejos et al., 2020). Another gene related with metals and increased in males is the metallothionein MT3, which may be secreted by astrocytes to reduce oxidative stress in neurons (Miyazaki et al., 2011; Z.-L. Wang et al., 2022). In addition, the increased expression of genes related to lipid metabolism such as FABP5 and APOD reinforce this hypothesis of protection, as they indicate a proper lipid homeostasis. APOD has also been described as both self-protective and protective of neurons in response to oxidative stimuli (Bajo-Grañeras et al., 2011; Najyb et al., 2017; Yin et al., 2021). These results are also consistent with those observed in our previous work by Lopez-Cerdan et al. in bulk RNA-seq, where we also detected processes related to metal homeostasis and apolipoproteins in males (López-Cerdán et al., 2022).

Meanwhile, female astrocytes presented functions involved in neurotransmitter reuptake, which could be interpreted as a protector mechanism against the excitotoxicity produced by glutamate spillover, one of the hallmarks of PD (Iovino et al., 2020). Among the genes involved, we highlight the solute transporter SLC1A2 (EAAT2) and the AMPA-type glutamate receptor GRIA2. EAAT2 is the main glutamate transporter expressed in astrocytes (Iovino et al., 2020; Liu et al., 2023) and protective overexpression has been reported in female mice with epilepsy (Hong et al., 2021). Likewise, GRIA2 overexpression has been described in females as neuroprotective in Alzheimer's disease (Chaudry et al., 2022) and in major depressive disorder (Gray et al., 2015), a predominant symptom in female PD (Meoni et al., 2020)

The predominance of protective processes in astrocytes of both sexes is not incompatible with the existence of reactive astrocytes that favor disease progression. The most likely explanation is that sex differences in astrocytes are mainly found in the phenotypes of the protective subpopulations of astrocytes, since transcriptomic studies in human patients and disease models have revealed the coexistence of many putative reactive sub-states of astrocytes (Patani et al., 2023).

The neuroinflammatory response is highly relevant in the development of PD, contributing directly and indirectly to oxidative stress and neuronal degeneration (Boyd et al., 2022; Mukhara et al., 2020). Together with astrocytes, microglia are the main cells involved in this process, and can adopt both detrimental and protective phenotypes in response to different stimuli (Terrin et al., 2023). In our results, male microglia are more active at the immune level, mainly related to an innate response, with functional terms related to macrophage activation and production of proinflammatory cytokines such as IL-12 or IL-17 (Figure 3C, E). The numerous infection-related pathways obtained in pathway analysis may also refer to this reactive phenotype (Figure 4A).

Female microglia, however, may have active processes such as tissue healing or projection formation, which may be involved in the degradation of protein aggregates by phagocytosis, whose capacity is greater in females even under physiological conditions (Yanguas-Casás, 2020). Numerous studies postulate the effect of female sex hormones as a possible etiology of the lower neuroinflammation in females (Cerri et al., 2019; Terrin et al., 2023; Yanguas-Casás, 2020). The overexpression of the PIBF1 gene (Progesterone Immunomodulatory Binding Factor 1) in female microglia could reinforce this theory, since it has been observed that some splice variants favor the production of immunosuppressive cytokines. (Raghupathy et al., 2009; Szekeres-Bartho et al., 2001).

Overall, male microglia is related to a greater immune response, particularly an innate-type response, while those of females may have a phenotype more oriented to repair and control of inflammation, probably due to the effect of hormones, coinciding with recent literature in this regard (Barko et al., 2022; Bourque et al., 2023; Villa et al., 2018).

Neurological processes related to PD also differ between sexes, with neurodegeneration being more present in males and axonal growth and guidance in females (Figure 3B-C, 4A). This neurodegenerative-like phenotype in males may be caused by the environment of increased neuroinflammation and excitotoxicity promoted by glial cells, which can trigger calcium-mediated apoptosis in neurons (Bartels et al., 2020; Flores-Cuadrado et al., 2021; Verma et al., 2022) and is overrepresented in our analyses (Figure 3B,E). In addition, various ubiquitins and phosphatases, detected as increased in male neurons (Figure 3G), might be related to PD pathogenesis, possibly due to a defect in mitophagy (Junqueira et al., 2019; Walden & Muqit, 2017). Relatedly, we identified an increase of expression in males of the genes SORL1 and VPS35, involved in the Retromer Complex (Figure 3G). These genes have been previously reported to be under-expressed in female mice with AD (Lane et al., 2010), and over-expression of VPS35 has also been linked to PD-related cellular stress and neuronal death (Tsika et al., 2014). This activation may come from the stimulatory interaction between the SORL1 receptor and APOE produced by oligodendrocytes (Figure 3H). Previously upregulation of both genes has been observed in myelinated neurons in early PD pathogenesis (Wilhelmus et al., 2011), although further studies are needed to establish the presence and importance of this interaction.

Nevertheless, we identified in female neurons a higher number of processes related to synapses and axon guidance than in male ones, mainly by signaling of the semaphorin-plexin system. This may respond to a potentiation of resilient neurons, probably due to estrogen effects (Y. H. Lee et al., 2019; Vegeto et al., 2019). In addition, we detected an increased expression of RET in female neurons, whose signaling is important in neuron protection and survival in PD models (Drinkut et al., 2016). It could also be acting on adhesion between neurons, since a truncated fragment of the protein can function as a cadherin accessory protein (Cabrera et al., 2011).

Previous studies with transcriptomic-based approaches and sex perspective have also related males with a phenotype more related to the pathological processes associated with Parkinson's disease (López-Cerdán et al., 2022; Simunovic et al., 2010). However, these sex-associated patterns of neurodegeneration and synapse potentiation are inverted in the cortical region results. In males, our results suggest a higher neuron connectivity in the cortical area, which have been described especially in patients with rigidity-dominant PD, also more common in males (Hou et al., 2017, 2018; Wichmann, 2019). We also observed in males processes related with a greater loss of neurons in the nigrostriatal system, existing bibliography that relate this to freezing of gait symptom, which is also more common in males (Bohnen et al., 2019; Kim et al., 2018). In contrast, our results suggest more neurodegeneration in cortical areas and a milder nigrostriatal impairment in female patients, being the former related to the tremor-dominant PD, more common among females (Haaxma et al., 2007). Furthermore, some studies have related dysfunction in some areas of the frontal cortex and a reduced anterior cingulate cortex with anxiety and depression states, also predominant symptoms in female PD patients (Carey et al., 2021; Laux, 2022). All this may indicate sex differences not only in the molecular mechanisms involved in disease progression but also in brain regions differentially affected by PD, with characteristic clinical implications.

In conclusion, we have identified different biological processes that outline a heightened immune response in males across both C and NS regions, and a greater neurodegenerative process in the NS in males compared to females, which seems to be inverted in the cortical region. Given the various approaches used, we identified signaling pathways, genes and cellular interactions involved in these differences, which may provide new insights to understand the molecular mechanisms of PD that can help to improve its diagnosis and personalized treatment.

We obtained these results through an *in silico* analysis of data available in public repositories, which demonstrates the power of using this type of approach to obtain more insight into the etiopathogenesis of different diseases from previously generated data. To promote these approaches, it is essential to conduct open research and make data available in public repositories according to FAIR principles (Findable, Accessible, Interoperable, Reusable), with full information on covariates of interest, such as sex. In this regard, a major pitfall in this meta-analysis was the inclusion of eligible studies with equal or similar representation of both sexes in each experimental condition. The most common scenario is the underrepresentation of females in this type of studies, which has been widely reported (Vaidya et al., 2021; Vitale et al., 2017; Weisman & Cassard, 1999). In our case, a representative example is the GSE157783 study, with only 1 female sample in each condition. This underrepresentation not only hinders *in silico* approaches as in our work, but can also introduce biases in the original studies, making it difficult to extrapolate the results

to general society. We consider it necessary to ensure the inclusion of sex information in the studies performed as well as the inclusion of an appropriate number of each of the sexes in the conditions analyzed.

We also acknowledge that C meta-analysis may present less robustness than NS meta-analysis, since it is composed of only 2 studies. Moreover, each study comes from a different cortical region (anterior cingulate cortex and prefrontal cortex), nevertheless, these regions are physically very close and closely related in the progression of the disease. (Braak et al., 2003; Del Tredici & Braak, 2016).

We tried to avoid these limitations by using a meta-analysis approach, an integration strategy that has never been used before in snRNA-seq datasets. This approach allows us to avoid biases arising from each individual study, and to work with relatively smaller datasets than if we tried to integrate all the data in one large dataset, increasing the reliability of the results and reducing the time and computational cost of the analyses. In addition, selecting the major cell types to perform the analyses allows us to work with a large number of cells from all the patients, which also increases the robustness of the results obtained. Finally, although sex-based meta-analyses of transcriptomic studies in this disease have been performed previously (López-Cerdán et al., 2022; Mariani et al., 2018), performing them on single cell technologies provides greater specificity and clarity of the results obtained. This could help to better interpret possible sex differences in PD by exploring the processes involved in each cell type, as well as the possible interactions between them.

In conclusion, this work has identified sex differences in potential genes, biological processes and pathways relevant to the pathogenesis of PD at cell type resolution. These results may help to better understand the clinical differences found between females and males and potentiate sex-based targeted therapy or drug repositioning research. As a final statement, we emphasize the importance of sharing data on open access platforms to facilitate scientific advancement.

Competing interests

The authors state no conflict of interest.

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