Nigral Neuroinflammation and Dopaminergic Neurons in Parkinson's Disease and Atypical Parkinsonisms

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Running head: Substantia nigra in parkinsonisms

Number of characters in the title: 89; Running head: 30
Number of words in abstract: 246; Introduction: 476
Number of words in discussion: 1831; Main text: 4411
Number of figures: 4; Tables: 3

SUMMARY FOR SOCIAL MEDIA IF PUBLISHED

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2. What is the current knowledge on the topic?

There has been growing interest in characterizing neuroinflammation in neurodegenerative diseases, as it may serve as a potential triggering factor inducing neuronal atrophy. Despite the evidence of increased neuroinflammatory activity from functional neuroimaging in individual conditions, there is a lack of comparative clinicopathological studies on this subject.

3. What question did this study address?

What is the interplay of neuroinflammation and clinical characteristics in PD and atypical parkinsonisms?

4. What does this study add to our knowledge?

Our findings reveal significant and distinctive T-cell-mediated neuroinflammatory activity within the SNc in PSP patients, which contrasts with the predominantly microglia-mediated neuroinflammation observed in PD patients. Moreover, both MSA and PSP patients demonstrated substantial losses of SNc dopaminergic neurons in comparison with PD patients.

5. How might this potentially impact on the practice of neurology?

The results highlight distinct neuroinflammatory patterns in the SNc among different parkinsonian disorders, indicating potential implications for disease progression and therapeutic strategies.

Abstract

Objective: To investigate the role of neuroinflammation in the substantia nigra pars compacta (SNc) across different parkinsonian disorders—Parkinson's disease (PD), progressive supranuclear palsy (PSP), and multiple system atrophy (MSA)—by examining SNc dopaminergic neuron counts, neuroinflammatory T cells, and microglial activity.

Methods: Postmortem neuropathological samples were collected from 79 individuals (PD, n=38; PSP, n=15; MSA, n=14; controls, n=12). The density of SNc tyrosine hydroxylase (TH)-positive neurons, T cells (CD3+, CD4+, CD8+), and Iba1 expression (Iba1-positive microglia/macrophages) were examined in the SNc and crus cerebri. Demographic and clinical data were gathered from patient histories.

Results: PSP patients had 89-212% more nigral CD3+, CD4+, and CD8+ T cells compared to MSA patients ($p<0.04$), 125-178% more CD3+ and CD4+ T cells than healthy controls ($p<0.002$), and 95% more CD4+ T cells than PD patients ($p=0.001$). Iba1 expression in the SNc was higher in PD patients than in MSA patients ($p=0.004$), with no significant differences observed across other conditions. There was a negative association between disease duration and SNc CD3+ T-cell density ($p=0.002$), and a positive correlation between nigral dopaminergic neuron density and CD3+ density, CD8+ density, and Iba1 expression in PD patients.

Interpretation: The study reveals distinctive neuroinflammatory patterns in the SNc, with T-cell-mediated inflammation prominent in PSP and microglia-mediated inflammation in PD. PSP and MSA show greater SNc dopaminergic neuron loss compared to PD. Increased neuroinflammatory response is seen in earlier disease stages, diminishing with greater neuron loss, which may inform disease progression understanding and therapeutic strategies.

Introduction

The substantia nigra (SN) plays a crucial role in the circuitry of the basal ganglia, regulating motor movement and the pathophysiology of parkinsonism.[1,2] The central causes of the motor symptoms observed in idiopathic Parkinson's disease (PD) are thought to be the gradual destruction of the nigrostriatal dopamine pathway from the SN pars compacta (SNc) to the striatum[1] and the accumulation of misfolded alpha-synuclein, resulting in the formation of Lewy bodies along this pathway and in other regions.[2] Current consensus within the field is that the motor symptoms observed in patients with PD, particularly bradykinesia and rigidity, predominantly arise from a pathological SNc, while the nonmotor symptoms of PD may be more closely associated with neuronal changes in other neurotransmitter systems.[3] Similarly, atypical parkinsonisms, including multiple system atrophy (MSA) and progressive supranuclear palsy (PSP), are generally characterized by a loss of SNc tyrosine hydroxylase (TH)-positive dopaminergic neurons.[4-7]

In recent years, there has been growing interest in characterizing neuroinflammation in neurodegenerative diseases, as it may serve as a potential triggering factor inducing neuronal atrophy.[8] Reports examining the brains of patients with PD have identified increased microglial activation in the SNc and other regions,[9] and the results of positron emission tomography (PET) studies have further suggested a connection between microglial activity and the pathological processes underlying PD.[10] Additionally, elevated levels of CNS-infiltrating T cells have been documented in the SNc of PD patients.[8, 11] Postmortem studies of the brains of MSA patients have similarly revealed microglial activation and T-cell infiltration, and these findings are supported by the results of related PET studies.[12-15] Likewise, in PSP, PET studies have indicated increased microglial activation in various brain regions.[16, 17]

Despite the evidence of increased neuroinflammatory activity from functional neuroimaging in individual conditions, there is a lack of comparative clinicopathological studies on this subject. Most in vivo and postmortem studies have primarily compared neuroinflammatory markers in patients with individual disorders with those in healthy

controls[8-11, 13, 15, 18], with some comparisons of either MSA or PSP with PD.[12, 19] Considering the inherent proteinopathic differences between synucleinopathies and 4R-tauopathies, as well as the unique phenotypic characteristics of the disorders, and acknowledging the suboptimal antemortem clinical accuracy in diagnosing degenerative parkinsonian disorders,[20] obtaining methodologically equivalent, pathologically confirmed comparative information is of high importance.

This study was designed to investigate and compare SNc dopaminergic and neuroinflammatory pathologies in PD, MSA and PSP patients. In a postmortem cohort comprising of 79 clinically well-characterized patients and healthy controls, we quantified dopaminergic neurons and CD3+, CD4+ and CD8+ T cells and measured the expression of the microglial marker Iba1 in the SNc of patients with different parkinsonian syndromes. We then conducted correlation analyses between these data and individual patient data related to phenotypic manifestations and disease progression. The identification of potential specific differences in neuroinflammatory profiles across these conditions could aid in the development of disease-specific neuroimmunological treatments.

Materials and methods

Subjects

The included subjects had undergone a neuropathological examination between 2002 and 2021 at the Department of Pathology, Turku University Hospital, Finland. The study was approved by the local ethical committee and was conducted according to the principles of the Declaration of Helsinki. The clinical histories of neuropathologically examined PD, PSP, and MSA patients were systematically reviewed by accessing the hospital's electronic medical record system or patient chart archive. Identification of the MSA and PSP subvariants was performed according to clinical diagnoses and phenotype descriptions. Checklists were utilized during the data collection process to ensure comprehensive analysis. Clinical symptoms or signs were considered present if they were recorded in the patient's records by the treating physician. A patient was classified as depressed if a code for an ICD-10 depression diagnosis (F32) was present in their record, the treating physician had documented depression at clinical visits, and/or the patient had been prescribed antidepressant medication used for mood disorders. A patient was categorized as having a sleep disorder if any nighttime condition that affected the quality, timing, or duration of sleep was present. These disorders included insomnia, REM sleep behavior disorder (RBD), and sleep apnea. For other symptoms and clinical features (Table 3), they were categorized as present if recorded in patient histories. Clinical data collected and classified included phenotypic details over the disease course; descriptive data related to rigidity, bradykinesia, resting tremor, and cognitive problems; the levodopa equivalent daily dose (LEDD, mg); and the Hoehn and Yahr (HY) stage[21].

Neuropathology

Immunohistochemistry experiments

The neuropathological methods used for assessing TH+ dopaminergic cells in the SNc have been described in detail previously.[22] Briefly, midbrain specimens were fixed in formalin, embedded in paraffin, and sectioned at 8 µm for TH staining and at 3.5 µm for other staining procedures (Supplementary Table 1 and Supplementary Methods).

TH-positive neurons in the SNc were manually and systematically counted from a single 8 µm section at high magnification. Since counting in two-dimensional sections can lead to errors due to overcounting, especially when counting objects that are large with respect to section thickness, neuron counts were corrected with the Abercrombie method,[23] as described previously,[22] which depends on section thickness and nuclear diameter along the appropriate axis.

Bioimaging analysis experiments involving model training

To evaluate T-cell numbers and densities and Iba1 expression, QuPath version 0.5.0 (https://qupath.github.io), an open-source software for bioimaging analysis, was used.[24] The SNc was delineated as the region of interest according to the outlines identified with Luxol Fast Blue or Substance P staining. To count T cells within these delineated regions, all cells were first detected by Stardist QuPath extension version 0.4 using the dsb2018_heavy_augment.pb model[25] in a modified version of Universal StarDist for QuPath script version 1.0.0.[26] In Stardist, cell detection was performed on the DAB colour channel after stain vector optimization. Any large neuromelanin-containing neurons detected were excluded by filtering the Stardist cell detections by nucleus size and stain intensity. A random forest object classifier was trained in QuPath to separate T cells from the remaining small neuromelanin fragments and other brown background features using a collage of training images annotated by a pathologist; these annotations included detection area size and shape descriptors, haematoxylin and DAB colour intensity descriptors and Stardist detection probabilities. Separate classifiers were trained for CD3, CD4, and CD8 staining, each of which was used to ultimately classify 79 images. After these exclusions, the remaining CD3+, CD4+ and CD8+ T cells were counted. For details, see Supplementary Methods.

Statistical Analyses

Statistical analyses were conducted using SPSS Statistics 29 for Macintosh (IBM Corp., Armonk, NY, USA). Nonparametric Kruskal–Wallis tests were used to assess differences in continuous variables among the four groups, with the post hoc Bonferroni correction for multiple comparisons. Categorical variables were assessed

using chi-square or Fisher's exact tests, and correlations between neuropathological measurements were examined by determining Spearman's correlation coefficients. Associations with the HY stage were not evaluated due to low variations in the values. To investigate clinical variables, an analysis of covariance (ANCOVA) model was applied, using logarithmic values for cell counts and densities and separately specifying the covariates for each analysis (sex, disease duration and/or group). For logarithmic values, β values with 95% confidence intervals were calculated. Possible interactions between covariates and groups were also assessed. Intraclass correlation coefficient (ICC) analyses were used to measure the reliability of the automated counting with respect to the manual method. The normality of the data was assessed using histograms and the Kolmogorov–Smirnov test. Values of $p<0.05$ were considered to indicate statistical significance. For tests correlating neuropathological measures with multiple clinical variables (age, sex, motor stage, disease duration and phenotype), a stricter statistical threshold of $p<0.01$ was employed.

Validation of Automated Counting

The intraclass correlation coefficient (ICC) for assessing the agreement between manual and automated counting of SNc CD3+ T cells for all 79 patients was 0.918 (95% CI 0.862-0.949), indicating that the results obtained using these two approaches were comparable. Similar ICC results were obtained when the manual and automated counting data for CD4+ and CD8+ T cells were compared (0.993 (95% CI 0.973-0.998) and 0.995 (95% CI 0.960-0.999), respectively).

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Results

Demographic and Clinical Characteristics

The study population comprised 79 participants (38 with neuropathologically confirmed PD, 15 with PSP, 14 with MSA, and 12 control subjects who died without a diagnosis of neurodegenerative disease). The primary demographic and clinical characteristics of the subjects are presented in Tables 1 and 2 and Figure 1. The median (range) time from death to autopsy was 4.5 (0-18) days (data were missing for 7 patients). All PD patients had correctly received a PD diagnosis antemortem based on their clinical phenotype and levodopa treatment response based on the UK Brain Bank criteria.[27] Six MSA patients had been diagnosed with MSA antemortem, three had been diagnosed with PD, four had been diagnosed with undetermined parkinsonism, and 1 had been clinically diagnosed with motor neuron disease. Among the PSP patients, six had been correctly diagnosed with PSP antemortem, five had been diagnosed with undetermined parkinsonism, two had a diagnosis of PD, and two had been diagnosed with corticobasal syndrome. In pairwise comparisons, PD patients were older at the time of death than patients in the other groups ($p<0.02$, Bonferroni corrected), while no significant group differences in sex distribution were identified. The median disease duration from the onset of motor symptoms to death was 5.5 years longer in PD patients than in MSA patients ($p=0.01$). Overall motor disease severity, as indicated by the HY scale score at the last evaluation before death, was greater in patients with PSP (median 5) and MSA (5) than in those with PD (4) ($p<0.01$). Furthermore, the last LEDD before death was higher in patients with PD than in those with PSP ($p=0.004$). Group differences related to motor phenotypes and asymmetry are presented in Table 3.

Lower SNc Dopaminergic Neuron Counts in PSP and MSA Compared to PD

Compared to healthy controls, patients with PSP or MSA and patients with PD had substantially fewer (by 72.5-78.3%) and moderately fewer (35.2%) TH+ neurons in the SNc, respectively ($p<0.05$ for both) (Tables 1 and 2, Figure 1). Furthermore, compared with the PD group, the PSP and MSA groups had 57.6% and 66.6% fewer SNc TH+

neurons, respectively ($p<0.05$ for both). The SNc TH+ neuron density was 50.5-78.1% lower in all patient groups than in the healthy control group ($p<0.02$), while no differences were observed among the PD, PSP and MSA groups. Similar results were observed when accounting for age and sex as covariates, with healthy individuals and PD patients having higher TH+ neuron counts and densities than PSP and MSA patients ($p<0.04$), while healthy controls had greater TH+ neuron densities than did PD patients ($p=0.03$).

Higher CD3+, CD4+ and CD8+ T Cell Counts in PSP Compared to Other Conditions

The nigral CD3+, CD4+ and CD8+ T-cell counts were substantially higher in PSP patients (89.4-212%) than in MSA patients ($p<0.04$). Furthermore, PSP patients had 125-178% more CD3+ and CD4+ T cells than did healthy controls ($p<0.002$) and 94.9% more CD4+ T cells than did PD patients ($p=0.001$); similar PSP-specific increases were observed in T-cell densities (Tables 1 and 2, Figure 2). When age and sex were considered as covariates, PSP patients still had higher CD3+ counts than did healthy controls ($p=0.045$), higher CD3+ densities than did PD patients and healthy controls ($p<0.031$), higher CD4+ counts and densities than did PD patients and healthy controls ($p<0.007$), and higher CD8+ counts than did MSA patients ($p=0.021$).

Higher Iba1 Expression in PD Compared to MSA

Compared with that in MSA patients, Iba1 expression in the SNc was markedly elevated (by 169%) in PD patients ($p=0.004$), while no significant differences were observed between the other pairs of groups. Moreover, extranigral Iba1 expression did not significantly differ across the different conditions (Tables 1 and 2, Figures 2 and 3). There were no significant group differences in SNc or extranigral Iba1 expression when age and sex were used as covariates, due to the older age of the PD group compared to the other groups ($p>0.56$).

Positive Association between TH+ Neurons and T Cells/Microglia in PD

Across the entire sample (n=79), neither the number or density of SNc TH+ neurons was significantly correlated with the number or density of SNc CD3+, CD4+ or CD8+ T cells. However, a weak positive correlation was observed between the number and density of SNc TH+ neurons and SNc Iba1 expression ($r>0.24$, $p<0.03$). In PD patients, positive correlations were observed between SNc TH+ neuron density and CD3+ T-cell density, CD8+ T-cell density, SNc Iba1 expression and crus cerebri Iba1 expression (Figure 4, Supplementary Table 2); no correlations were observed between these measures in the other groups. Robust positive correlations were detected among CD3+, CD4+ and CD8+ T-cell counts and densities ($r>0.43$, $p<0.001$), as well as between nigral and extranigral Iba1 expression ($r>0.73$, $p<0.001$). Furthermore, lba1 expression in both the SNc and crus cerebri was positively correlated with SNc CD8+ T-cell density ($r=0.29$, $p=0.009$; $r=0.34$, $p=0.002$), while no such correlations were observed with the other nigral T cells.

Negative Association between Disease Duration and CD3+ T Cells Across Conditions

Demographic and clinical characteristics were investigated to determine if they are associated with the neuropathological measures obtained in the study. Age at death showed no significant association with the count or density of TH+ neurons, T cells or microglia ($p>0.41$, group as covariate; age*group interaction $p>0.058$). However, across groups, male sex was associated with higher extranigral Iba1 expression ($\beta=0.55$, 95% CI = 0.20 to 0.91, $p=0.003$, group as covariate; sex*group interaction $p=0.20$), higher SNc CD3+ T-cell count ($\beta=0.46$, 95% CI = 0.14 to 0.78, $p=0.005$, group as covariate; sex*group interaction $p=0.83$) and higher SNc CD4+ T-cell count ($\beta=0.53$, 95% CI = 0.22 to 0.84, $p=0.001$, group as covariate; sex*group interaction $p=0.59$). Consequently, sex was included as a covariate in subsequent analyses of these measurements.

Disease duration from symptom onset to death was negatively associated with SNc CD3+ T-cell density across groups ($\beta=-0.063$, 95% CI = -0.10 to -0.02, $p=0.002$, group

as covariate; group*disease duration interaction p=0.077) and with SNc CD3+ T-cell count across groups (β=-0.065, 95% CI = -0.10 to -0.03, p=0.002, group and sex as covariates; group*disease duration interaction p=0.24) (Figure 4). With group as the covariate, the group*disease duration interaction effect was significant for SNc CD8+ T-cell density (p=0.06, interaction p=0.004) and count (p=0.045, interaction p=0.004), while no associations were observed for the other cell types (p>0.10, interaction p>0.27). However, in separate subgroup analyses, the association between SNc CD8+ T-cell density/count and disease duration was not significant (PD p>0.60, PSP p>0.11, MSA p>0.050; no covariates). Disease duration was used as a covariate in subsequent analyses involving SNc CD3+ and CD8+ T-cell densities and counts.

Negative Association between Depression and TH+ neurons Across Conditions

Depression (β=-0.53, 95% CI = -0.88 to -0.17, p=0.004, group as covariate; group*depression interaction p=0.71) and sleep disorders (β=-0.44, 95% CI = -0.77 to -0.11, p=0.009, group as covariate; group*sleep disorders interaction p=0.50) showed negative associations with SNc TH-positive neuron density across patient groups. Similar associations were observed between TH-positive neuron count and depression (β=-0.52, 95% CI = -0.87 to -0.18, p=0.003; group as covariate; group*depression interaction p=0.42; Figure 4). Across groups, patients with depression had more sleeping problems than did patients who did not have depression (58.8% vs. 24.0%, p=0.008). No other significant phenotypic associations were identified. There were no differences in neuronal or neuroinflammatory marker levels between MSA-P (n=11) and MSA-C (n=3) patients (p>0.23) or between PSP-RS (n=10) and less common PSP variants (n=5, p>0.25).

Discussion

The primary objective of this study was to investigate the interplay between nigral dopaminergic degeneration and neuroinflammation across various parkinsonian disorders. The key findings from these clinicopathological examinations are: 1) Despite the shorter disease durations of MSA and PSP, substantially fewer nigral dopaminergic neurons were observed at the time of death in patients with these conditions than in those with PD, indicating a clearly accelerated dopaminergic neurodegenerative process; 2) PSP patients demonstrated a distinctive increase in nigral T cells relative to PD and MSA patients and neurologically healthy controls, signifying a specific and marked end-stage neuroinflammatory response unique to PSP; 3) PD was linked to increased microglia activity, suggesting a distinct microglia-driven nigral neuroinflammatory process in this disorder; and 4) the neuroinflammatory activity observed in degenerative parkinsonisms appeared to decrease over time, particularly in cases of CD3+ T-cell infiltration. Moreover, in PD, nigral neuroinflammation decreased as the severity of nigral dopaminergic neuron loss increased.

PSP and MSA are associated with a markedly greater loss of nigral dopaminergic neurons than PD

Both PSP and MSA patients showed significant reductions in SNc dopaminergic neurons (by 58-67%) relative to PD patients. Furthermore, an intermediate reduction in the number of dopaminergic neurons was observed in PD patients compared to individuals without neurodegenerative disorders. These reductions are consistent with the known shared vulnerability of dopaminergic neurons under these conditions, as seen in dopaminergic functional brain imaging.[28, 29] However, despite having notably shorter disease durations than PD, both PSP and MSA were associated with a markedly lower number of dopaminergic neurons. To our knowledge, only one prior study has compared the number of nigral dopaminergic neurons in PD, PSP and MSA patients (total n=35), but the semiquantitative grading system (mild/moderate/severe) did not show differences in nigral cell loss.[30] Studies have suggested a more widespread loss of nigral A10 dopamine neurons in PSP than in PD[31, 32], whereas the

loss of A9 dopamine neurons has been comparable[6]. A previous postmortem study reported 55% greater nigral neuronal loss in MSA patients than in healthy controls.[18]

PSP is linked to distinctive nigral T-cell infiltration

Another notable and novel observation was the clear elevation (by 89.4% to 212%) in the CD3+, CD4+, and CD8+ T-cell count and density within the SNc of PSP patients at the time of death relative to MSA and PD patients and healthy controls. This finding suggests the presence of PSP-specific neuroinflammatory activity which could be related to pathophysiological differences between tauopathies and synucleinopathies.[33] An essential question arising from this observation is whether this increased neuroinflammatory activity in PSP is a specific late-stage phenomenon or if it is already evident during the early stages of the disease. Although the current study cannot definitively address this question, it is crucial to note that no differences were observed between PSP and MSA patients in terms of nigral dopaminergic neuronal loss, demographics or factors related to disease severity. Nevertheless, PSP patients had much higher CD3+, CD4+ and CD8+ T-cell counts than did MSA patients, suggesting that neither general disease severity nor nigral neuronal loss can explain the increase in T cells.

An additional observation challenging the hypothesis of T-cell infiltration as a late-stage phenomenon is the inverse relationship between disease duration from symptom onset to death and the number of CD3+ T cells in the SNc across groups. This finding suggests that CNS T-cell infiltration is not a cumulative process that solely manifests in the advanced stages of the disease; rather, nigral T-cell infiltration, particularly CD3+ T-cell infiltration, may be an earlier pathophysiological event, and overall neuroinflammatory activity tends to decrease over the course of the disease. This is further corroborated by the positive correlation observed between nigral TH+ neuron counts and densities and multiple neuroinflammatory markers in PD patients, suggesting that inflammation is less pronounced in individuals with advanced nigral neuronal loss. An alternative explanation for this finding is that patients with high T-

cell counts have more aggressive disease, leading to faster disease progression and death.

Importantly, while PSP patients presented with greater T-cell numbers, Iba1 expression, which reflects microglial/macrophage activity, was not significantly different between the PSP group and the other groups; however, PD patients showed greater Iba1 expression than MSA patients, which is generally in line with previous data in PD patients.[8, 34-37] It is possible that increased microglial activity is also present in early stages of PSP, yet the severe degenerative process associated with this disease might subsequently deactivate microglia, leaving an overactive T-cell-mediated neuroinflammatory response that persists in advanced stages. However, notably, there were no differences in SNc Iba1 expression among PD patients, MSA patients and healthy controls in our study. Although this finding contradicts that of the seminal study by McGeer et al in 1988,[8] other investigations have reported similar findings regarding PD.[10, 38]

Our findings also seem to contradict those of a study that revealed a greater number of microglia in the SNc of eleven patients with MSA than in that of healthy controls.[18] Furthermore, a recent PET imaging study revealed increased translocator protein expression by glia in the lentiform nucleus and cerebellar white matter in individuals with MSA compared to those with PD[39]. However, the interpretation of microglial activation using the PET signal for translocator protein is debatable since it may not be specific[40]. Methodological differences in microglial identification, patient populations (early vs. advanced disease) and regional specificity of neuroinflammatory processes may account for these different findings.

Regarding the roles of specific T cells in our findings, CD4+ T cells, primarily involved in helper functions, play a crucial role in regulating innate and adaptive immune responses[41]. The increased CD4+ T cell density in PSP may reflect a heightened immune activation, consistent with the unique neuroinflammatory profile of this disorder. Conversely, CD8+ T cells, which are cytotoxic, are often involved in targeting and eliminating damaged or infected cells[42], and their elevated density in PSP

suggests a possible immune response to neuronal damage. These findings highlight the differential roles of T cell subtypes in the pathophysiology of parkinsonian disorders, with PSP showing a stronger adaptive immune response and PD exhibiting a more prominent microglial-driven innate immune response.

While T-cells and microglia both contribute to neuroinflammation, our findings suggest they operate through distinct mechanisms in PD and PSP. In PSP, T-cell-mediated inflammation predominates, while in PD, microglia-driven neuroinflammation is more prominent. The observed correlations between T-cell density and microglial activity may indicate an interaction between these immune cell types, yet their respective roles in neurodegeneration appear to differ in the two disorders. While microglia-mediated T-cell infiltration appears central to neurodegeneration in tauopathies[43], expanding the analysis to include additional neuroinflammatory markers, such as B lymphocytes and astrocytes, will be crucial to better understanding their potential roles in disease progression. Future studies should also employ more specific markers, such as P2Y12 or TMEM119, to distinguish microglia from infiltrating macrophages. Additionally, skeletonized analysis and phenotyping of microglial cells should be considered to better understand the different activation states of microglia across parkinsonian disorders and to explore potential disease-specific differences in microglia responses. Although these analyses were beyond the scope of the current study, they will provide a valuable direction for future research.

Associations with phenotype

In the investigation of clinical symptomatology among patients, we observed a link between depression and sleep disorders and decreases in the count and density of SNc dopaminergic neurons across all patient groups. Our previous findings, based on a partially overlapping dataset, established a connection between depression and the number of SNc neurons in PD patients.[22] The current results extend this association to atypical parkinsonisms, which are frequently linked to depressive symptoms.[44] However, the effect was less pronounced in atypical parkinsonisms compared to PD. It is important to interpret these results cautiously, given that the association between depression and sleep disorders is well-documented and might contribute to this observed correlation.

With regard to T-cells or Iba1 expression, no significant associations with symptomatology were identified. This lack of association may be attributed to the categorical nature of the clinical data, the reliance on patient histories, and the advanced stage of our patient cohort, characterized by various severe and mixed nonmotor symptoms. To further elucidate these relationships, future studies should be conducted in patients in earlier disease stages while leveraging PET or CSF neuroinflammatory markers.

Limitations and strengths

We recognize certain limitations in the interpretation of our findings. First, our observations are reflective of end-stage pathology, and thus, we cannot definitively conclude whether the observed changes occur solely at the terminal phase of the disease or whether they manifest years before symptom onset. However, the absence of demographic and clinical differences between MSA and PSP patients despite the presence of substantial differences in neuroinflammatory activity, and the inverse relationship between T-cell count and disease duration, suggest that our are not driven by disease severity or symptom duration. Second, our investigation was limited to midbrain sections. Future studies should consider additional striatal, cortical, and cerebellar regions to produce a more comprehensive understanding of neuroinflammatory pathology in these diseases. Third, we did not observe differences in neuroinflammation, or dopaminergic neuron counts among different MSA and PSP disease subtypes because of the limited sample size of MSA-C patients and patients with less common PSP variants; this underscores the need for further investigations with larger numbers of patients. Fourth, the retrospective nature of clinical data collection from patient histories prevented the use of validated numerical clinical scales that would have provided information about symptom severity. This limitation should be considered when interpreting the associations between cell counts and clinical symptoms.

Finally, a methodological limitation of our study is the use of Abercrombie correction for TH+ neuron counting, which, while helpful, does not fully replace unbiased stereological methods. Stereological counting remains the gold standard for accurate cell enumeration and provides a more reliable estimate of neuron density. However,

typically this method requires tissue processing that was not feasible with the formalin-fixed, paraffin-embedded samples. Instead, we manually counted TH+ dopaminergic neurons from a large area (the entire SN, both right and left) using 8-micrometer single sections. While stereological counting in the human SN typically requires a limited tissue cube, our approach covers a much broader area in the horizontal plane. The Abercrombie method is area-based rather than volume-based, and we acknowledge that this methodological constraint may affect the precision of our results, and future studies employing stereological techniques could validate and refine our finding**.** Despite these limitations, our study has notable strengths. The inclusion of four comparative groups, complete diagnostic certainty, validated cell counting, and the utilization of multiple neuropathological markers contributed to the robustness of our findings.

Conclusions

The trajectory from symptom onset to death in patients with PD, MSA, or PSP is characterized by a profound loss of nigral dopaminergic neurons, with atypical parkinsonisms clearly exhibiting more pronounced degeneration. Neuroinflammation in these disorders is characterized by distinct patterns: PSP exhibits a T-cell-mediated inflammatory response, whereas PD is associated with primarily microglia-driven neuroinflammation. These findings underscore that neuroinflammation is a dynamic and disease-specific phenomenon, reflecting different inflammatory pathways in each disorder.

Acknowledgements

The authors would like to express their gratitude to the staff of the Department of Pathology and Neurocenter at Turku University Hospital for their valuable support. We express our gratitude to Sinikka Kollanus for her technical assistance. The research reported in this publication was supported by The Finnish Parkinson Foundation, The Päivikki and Sakari Sohlberg Foundation and Turku University Hospital (VTR-funds).

Author contributions

VK and MG contributed to the conception and design of the study; EB, MG, LL, MP, TV, and VK contributed to the acquisition and analysis of data; EB, MG, LL, MP, TV, PB, NS, GW and VK contributed to drafting the text and preparing the figures.

Potential conflicts of interest

The authors report no competing interests.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. Clinical and neuropathological group differences. (A) Age at death, (B) Disease duration from motor symptom onset to death, (C) Levodopa equivalent daily dose (LEDD) of dopaminergic medications (last recorded value before death), (D) Brain weight at neuropathological examination, (E) Substantia nigra pars compacta (SNc) tyrosine hydroxylase (TH)+ neuron count, and (F) SNc TH+ neuron density across patient groups. *p<0.05, **p<0.01, ***p<0.001. PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy, HC = healthy controls.

Figure 2. Neuroinflammatory markers in parkinsonian disorders. (A) CD3+ T-cell count, (B) CD3+ T-cell density, (C) CD4+ T-cell count, (D) CD4+ T-cell density, (E) CD8+ T-cell count, (F) CD8+ T-cell density, (G) SNc Iba1 expression (microglial activation), and (H) Crus cerebri Iba1 expression. *p<0.05, **p<0.01, ***p<0.001. PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy, HC = healthy controls.

Figure 3. Representative histopathological sections of SNc from different patient groups. (A) A 76-year-old male patient diagnosed with progressive supranuclear palsy (PSP), disease duration 7.5 years, (B) A 65-year-old female patient diagnosed with multiple system atrophy (MSA), disease duration 4.0 years, (C) A 76-year-old male patient diagnosed with Parkinson's disease (PD), disease duration 13.3 years, and (D) A 72-

year-old male control without a degenerative parkinsonian disorder. Brown staining in the first column highlights neuromelanin-laden dopaminergic neurons in the substantia nigra. The immune cells (CD3+, CD4+, CD8+) are shown at 60x magnification in the following columns (arrows), and the brown staining in the last two columns indicates Iba1 expression (arrows).

Figure 4. Associations between neuroinflammation, depression, and disease duration.

(A) Comparison of SNc TH+ dopaminergic neuron density in patients with and without depression. (B) Correlation between disease duration and CD3+ T-cell density across patient groups. (C) Relationship between nigral TH+ neuron density and Iba1 expression (microglial activity) in PD patients. *p<0.05, **p<0.01. PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy.

A

Age at death (years)

100
80
60

*
**
*

PD PSP MSA HC

B

Disease duration (years)

25
20
15
10
5
0

*

PD PSP MSA

C

LEDD (mg)

1500
1000
500
0

**

PD PSP MSA

D

Brain weight (g)

1800
1500
1200
900

PD PSP MSA HC

E

SNc TH+ neuron count (n)

250
200
150
100
50
0

*
***
**
*
***

PD PSP MSA HC

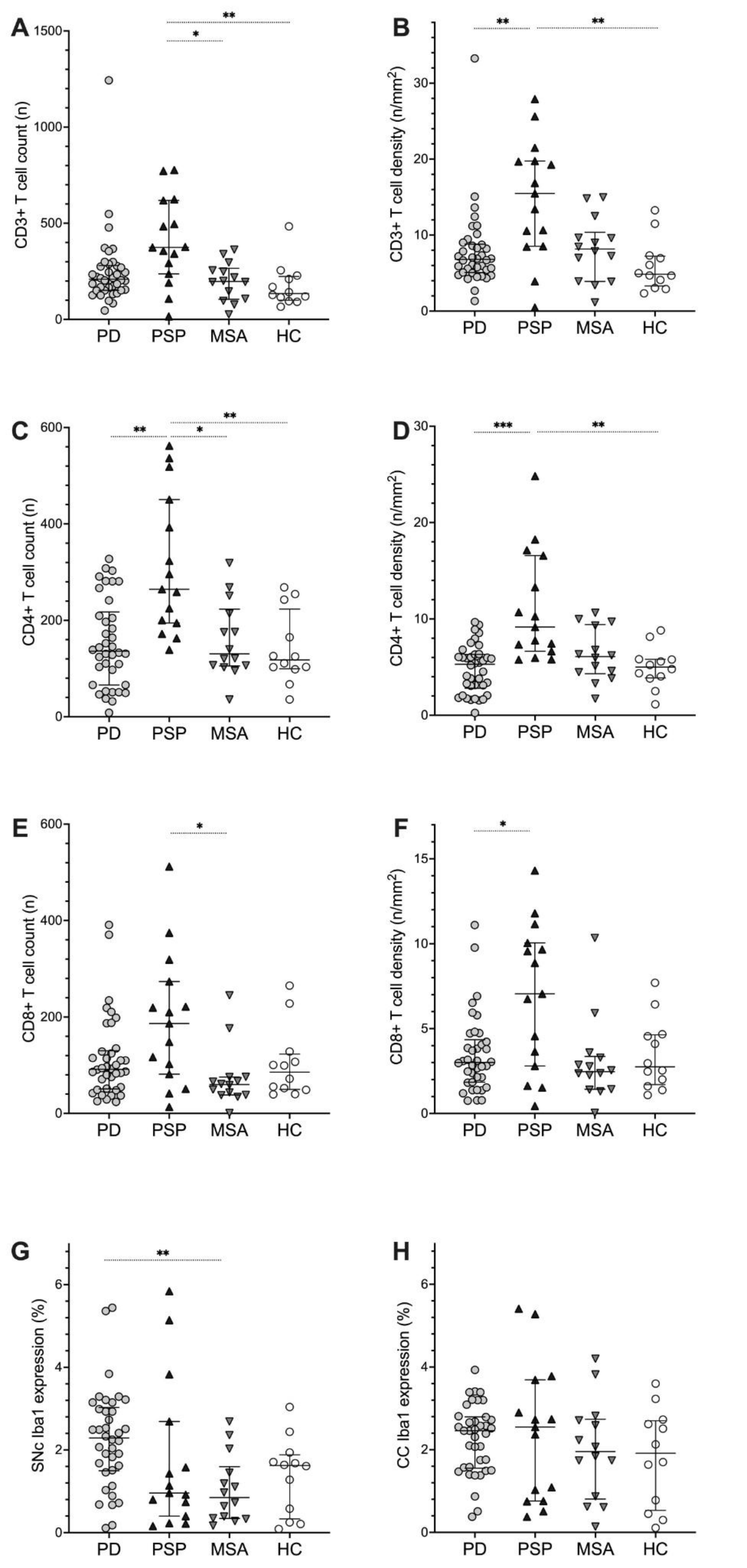
F

SNc TH+ neuron density (n/mm^2)

6
4
2
0

*
***
***
***

PD PSP MSA HC


A
CD3+ T cell count (n)
1500
1000
500
0
**
*
PD
PSP
MSA
HC
B
CD3+ T cell density (n/mm2)
30
20
10
0
**
**
PD
PSP
MSA
HC
C
CD4+ T cell count (n)
600
400
200
0
**
*
**
PD
PSP
MSA
HC
D
CD4+ T cell density (n/mm2)
30
20
10
0
***
**
PD
PSP
MSA
HC
E
CD8+ T cell count (n)
600
400
200
0
*
PD
PSP
MSA
HC
F
CD8+ T cell density (n/mm2)
15
10
5
0
*
PD
PSP
MSA
HC
G
SNc Iba1 expression (%)
6
4
2
0
**
PD
PSP
MSA
HC
H
CC Iba1 expression (%)
6
4
2
0
PD
PSP
MSA
HC

CD3+ CD4+ CD8+ Iba1+ SNc Iba1+ Crus Cerebri

A

B

C

D

PSP

MSA

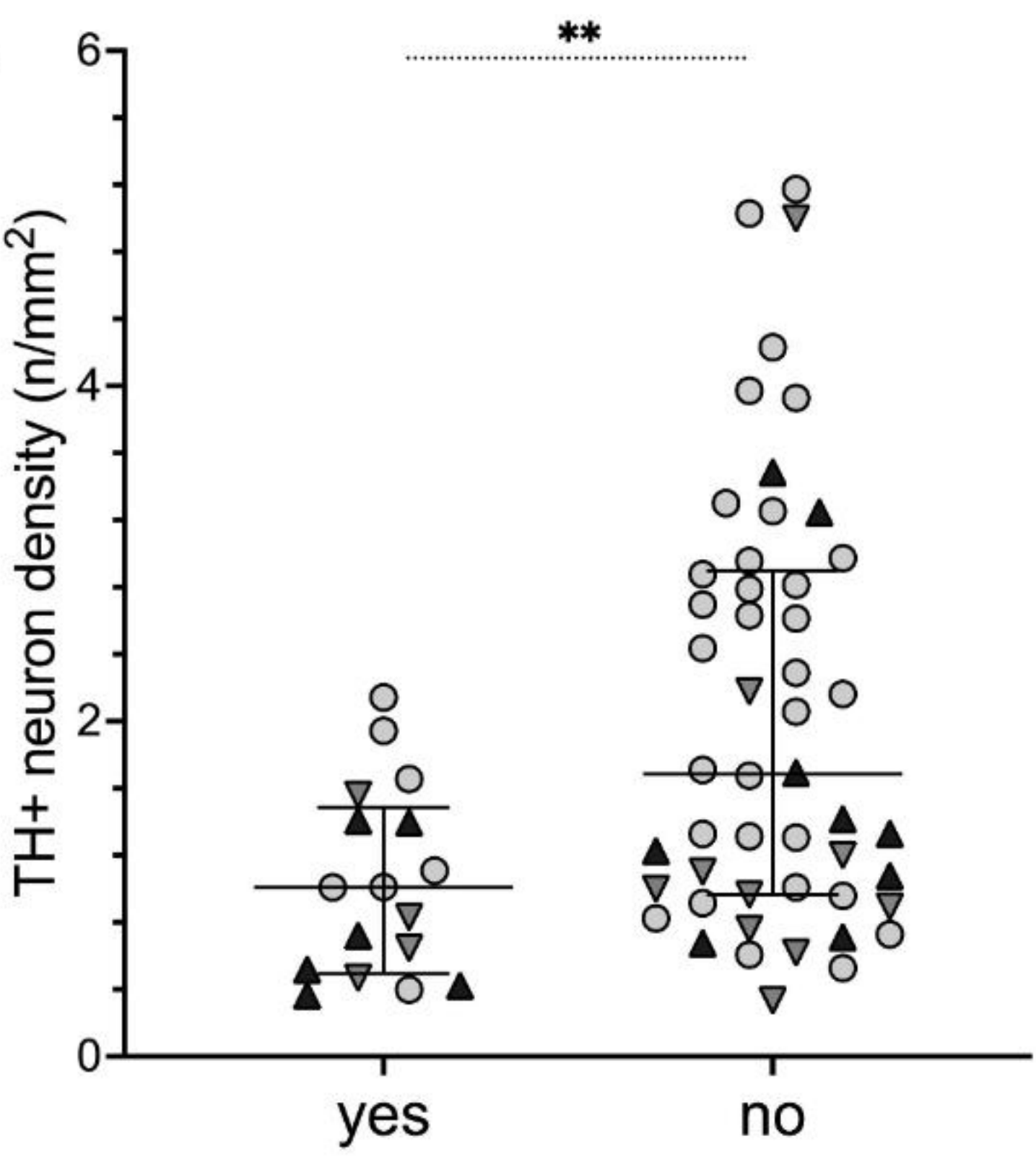
PD

HC

50 μm

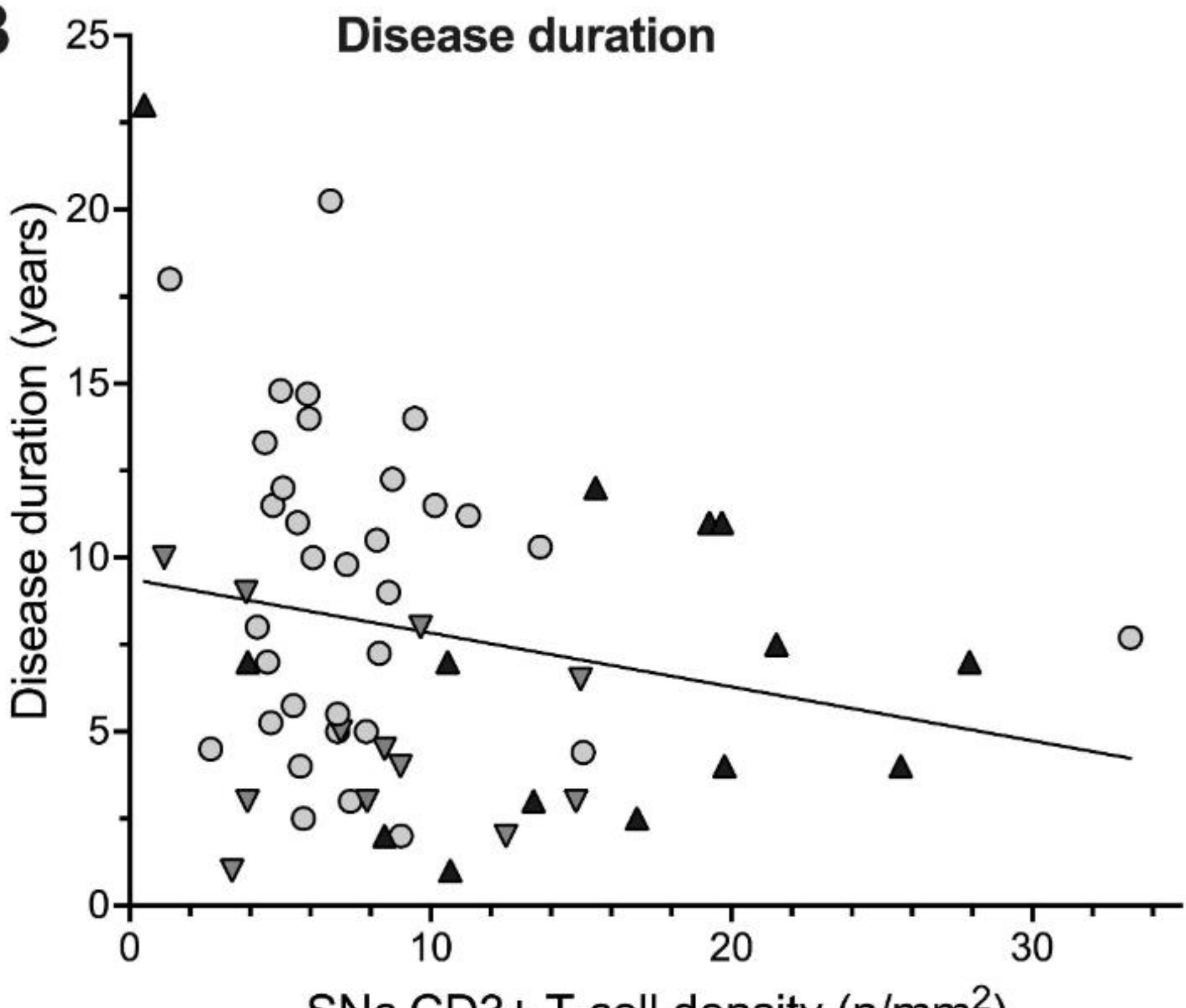
A

Depression



B

Disease duration



C

TH+ neuron density vs microglia

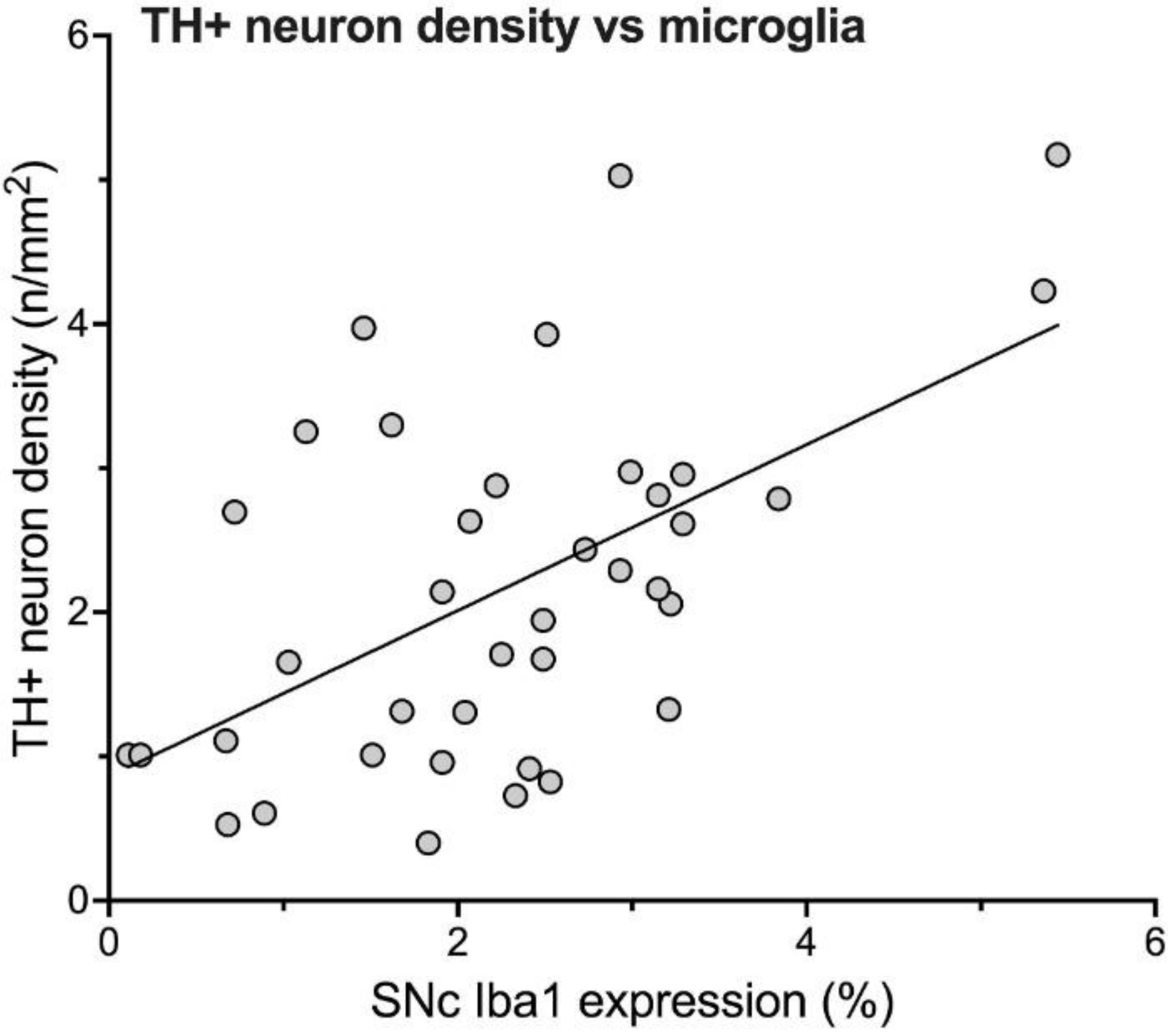


Table 1. Group differences in baseline demographic, clinical, and neuropathological characteristics. Data are presented as the median [interquartile range] or n.

Variable group	Variable		Group				*p* value
			PD	PSP	MSA	HC	
Demographics	Number of patients		38	15	14	12	-
	Age at death (years)		80.3 [9.2]	73.6 [8.0]	70.3 [19.2]	69.5 [16.7]	<0.001
	Sex (m/f)		30/8	9/6	6/8	7/5	ns
Clinical characteristics	Disease duration from motor symptom onset to death (years)		9.8 [7.0]	7.0 [8.1]	4.3 [4.6]	-	0.009
	HY score (last before death)		4 [2]	5 [1]	5 [0]	-	<0.001
	LEDD at death (mg)		574 [455]	0 [520]	496 [1039]	-	0.006
	Motor phenotype (tremor/no tremor)		24/7	5/10	7/7	-	0.011
	Asymmetry of motor symptoms (asymmetrical/symmetrical)		26/0	5/6	0/11	-	<0.001
Nigral neuropathology	SNc TH+ neuron count (n)		70.0 [43.2]	29.7 [31.3]	23.4 [18.7]	108 [54.8]]	<0.001
	SNc area (mm^2)		31.0 [10.8]	25.4 [7.9]	23.7 [6.76]	28.8 [11.1]	0.002
	SNc TH+ neuron density (corrected n/mm^2)		2.10 [1.89]	1.23 [0.74]	0.93 [0.65]	4.24 [1.0]	<0.001
	SNc asymmetry index (%)[A]		80.1 [30.0]	69.5 [27.8]	53.5 [38.5]	78.7 [27.0]	ns
	Brain weight (g)		1420 [218]	1366 [116]	1373 [276]	1398 [239]	ns
	Death to autopsy delay (days)		5.5 [4]	3 [7]	4 [2]	3 [5]	ns
Neuroinflammation markers	CD3+	Count (n)	209 [129]	375 [382]	198 [161]	135 [124]	0.003
		Area (mm^2)	32.2 [10.3]	27.6 [9.8]	24.0 [7.1]	29.6 [12.5]	0.01
		Density (n/mm^2)	6.80 [3.73]	15.5 [11.2]	8.17 [6.47]	4.84 [3.92]	0.002
	CD4+	Count (n)	136 [152]	265 [256]	131 [118]	118 [124]	<0.001
		Area (mm^2)	31.3 [8.9]	29.6 [9.1]	25.4 [7.4]	27.8 [8.0]	0.02
		Density (n/mm^2)	5.30 [3.51]	9.16 [9.92]	6.01 [5.10]	5.01 [1.94]	<0.001
	CD8+	Count (n)	91.8 [79.3]	187 [192]	60.0 [37.3]	85.8 [73.3]	0.018
		Area (mm^2)	33.3 [10.8]	27.6 [7.9]	24.7 [6.7]	29.0 [10.4]	0.001
		Density (n/mm^2)	3.01 [2.51]	7.05 [7.25]	2.46 [1.94]	2.75 [2.93]	0.03
	Microglia	SNc Iba1 expression (%)	2.29 [1.53]	0.96 [2.29]	0.85 [1.26]	1.63 [1.55]	0.004
		Crus cerebri Iba1 expression (%)	2.46 [1.24]	2.55 [2.93]	1.96 [1.93]	1.91 [2.17]	ns

PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy, HC = control without degenerative parkinsonism, HY = Hoehn and Yahr, LEDD = levodopa equivalent daily dose, SNc = substantia nigra pars compacta, TH = tyrosine hydroxylase, ns = non-significant

P values are from the Kruskal–Wallis test, chi–square test or Fisher's exact test.

[A] (lower/higher side neuron count) × 100

Table 2. Pairwise group differences following the post hoc **Bonferroni correction**

Variable group	Variable		PD vs. PSP	PD vs. MSA	PD vs. HC	PSP vs. MSA	PSP vs. HC	MSA vs. HC
Demographics	Age at death (years)		0.018	0.001	0.011	ns	ns	ns
	Sex (m/f)		ns	ns	ns	ns	ns	ns
Clinical characteristics	Disease duration from symptom onset (years)		ns	0.01	-	ns	-	-
	HY score (last before death)		0.006	<0.001	-	ns	-	-
	LEDD at death (mg)		0.004	ns	-	ns	-	-
	Motor phenotype (tremor/no tremor)		0.028	ns	-	ns	-	-
	Predominant side of motor symptoms (right/left/symmetrical)		0.005	<0.001	-	ns	-	-
Nigral neuropathology	SNc TH+ neuron count (n)		0.04	0.003	0.048	ns	<0.001	<0.001
	SNc area (mm^2)		ns	0.002	ns	ns	ns	ns
	SNc TH+ neuron density (corrected n/mm^2)		ns	ns	0.015	ns	<0.001	<0.001
	SNc asymmetry index (%)[A]		ns	ns	ns	ns	ns	ns
	Brain weight (g)		ns	ns	ns	ns	ns	ns
	Death to autopsy delay (days)		ns	ns	ns	ns	ns	ns
Neuroinflammation marker expression	CD3	Count (n)	ns	ns	ns	0.037	0.003	ns
		Area (mm^2)	ns	0.007	ns	ns	ns	ns
		Density (n/mm^2)	0.007	ns	ns	ns	0.002	ns
	CD4	Count (n)	0.001	ns	ns	0.018	0.004	ns
		Area (mm^2)	ns	0.013	ns	ns	ns	ns
		Density (n/mm^2)	<0.001	ns	ns	ns	0.003	ns
	CD8	Count (n)	ns	ns	ns	0.010	ns	ns
		Area (mm^2)	ns	0.001	ns	ns	ns	ns
		Density (n/mm^2)	0.034	ns	ns	ns	ns	ns
	Microglia	SNc Iba1 expression (%)	ns	0.008	ns	ns	ns	ns
		Crus Cerebri Iba 1 expression (%)	ns	ns	ns	ns	ns	ns

PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy, HC = control without degenerative parkinsonism, HY = Hoehn and Yahr, LEDD = levodopa equivalent daily dose, SNc = substantia nigra pars compacta, TH = tyrosine hydroxylase, ns = non-signinicant

Table 3. Clinical characteristics of the studied PD, PSP and MSA patients Data are presented as n.

Variable	Group			*p* value	*Post hoc*		
	PD	PSP	MSA		PD vs. PSP	PD vs. MSA	PSP vs. MSA
n	38	15	14	-	-	-	-
Sleep disorder (y/n)	10/28	6/9	6/8	ns	ns	ns	ns
Depression (y/n)	7/31	6/9	4/10	ns	ns	ns	ns
Hyposmia (y/n)	6/32	0/15	0/14	ns	ns	ns	ns
Orthostatic hypotension (y/n)	14/24	1/14	8/6	0.015	ns	ns	**
Constipation (y/n)	12/26	4/11	7/7	ns	ns	ns	ns
Urinary incontinence (y/n)	12/26	4/11	8/6	ns	ns	ns	ns
Urinary retention (y/n)	7/31	6/9	4/10	ns	ns	ns	ns
Dysphagia (y/n)	8/30	8/7	9/5	0.006	ns	*	ns
Antipsychotic drugs (y/n)	18/20	3/12	0/14	0.001	ns	*	ns
Hallucinations (y/n)	19/19	3/12	0/14	<0.001	ns	**	ns
Dysarthria or speech difficulties (y/n)	10/28	12/3	12/2	<0.001	**	**	ns

PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy, ns = non-significant, *p<0.05, **p<0.01, ***p<0.001

P values denote the chi-square test or Fisher's exact test and post hoc tests corrected for multiple comparisons according to the Bonferroni method.

Supplementary Table 1. Summary of immunohistochemical methods.

Antibody, source	clone/catalogue number	Dilution	Autostainer	Detection
Substance P (Abcam)	EPR3959/ab133240	1:100	Labvision (Thermo-Fisher Scientific, Fremont, CA, USA)	BrightVision DPVB110HRP + BrightDAB (WellMed, Duiven, the Netherlands)
TH (Novocastra, Newcastle, UK)	1B5/NCL-TH	1:50	Labvision	BrightVision DPVB110HRP + BrightDAB
CD3 (Ventana Co., Tucson, AZ, USA)	2GV6/CONFIRM CD3 790-4341	ready to use	Ventana Bench Mark ULTRA (Ventana)	Ultraview IHC DAB (Ventana)
CD4 (Ventana)	SP35/CONFIRM CD4 790-4423	ready to use	Ventana Bench Mark ULTRA	Optiview IHC DAB, Ventana
CD8 (Novocastra)	4B11/NCL-L-CD8-4B11	1:100	Ventana Bench Mark ULTRA	Optiview IHC DAB
Iba1 (Cell Signaling Technology, Danvers, MA, USA)	AIF-1/17198	1:2000	Labvision	BrightVision DPVB110HRP + BrightDAB

TH = tyrosine hydroxylase, DAB = diaminobenzidine

Supplementary Table 2. Correlations between TH+ neuron density and neuroinflammatory markers.

	PD	PSP	MSA	HC	All
CD3+ T-cell density	0.41*	-0.029	0.28	-0.46	-0.046
CD4+ T-cell density	0.15	-0.20	0.46	-0.55	-0.14
CD8+ T-cell density	0.46**	-0.15	-0.007	0.070	0.098
SNc Iba1 expression	0.47**	0.079	0.13	0.049	0.25*
CC Iba1 expression	0.33*	-0.007	0.06	0.13	0.06

PD = Parkinson's disease, PSP = progressive supranuclear palsy, MSA = multiple system atrophy, HC = control without degenerative parkinsonism, SNc = substantia nigra pars compacta, CC = crus cerebri

Spearman correlation (R) * $p<0.05$, ** $p<0.01$, *** $p<0.001$.