Concomitant AD and DLB pathologies shape subfield microglia responses in the hippocampus

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Running title:

Hippocampal microglia and proteinopathies

Keywords:

Alzheimer's Disease; Dementia with Lewy Bodies; Hippocampus; Microglia; Amyloid-beta, Tau, Alpha-synuclein.

Number of Figures: 6

Number of Supplementary Figures: 8

Number of Pages: 31

Abbreviations:

A β = Amyloid- β ; AD = Alzheimer's Disease; APP = Amyloid precursor protein; CA = *Cornu ammonis*; CTL = Control; DG = *Gyrus dentatus*; DLB = Dementia with Lewy Bodies; MIC-MAC = Microglia and Immune Cells Morphologies Analyser and Classifier; PMD = Post-mortem delay; P-Syn = Hyperphosphorylated α -synuclein; P-Tau = Hyperphosphorylated tau; UMAP = Uniform manifold approximation and projection.

Abstract

Hippocampal alteration is at the centre of memory decline in the most common age-related neurodegenerative diseases: Alzheimer's disease (AD) and Dementia with Lewy Bodies (DLB). However, the subregional deterioration of the hippocampus differs between both diseases with more severe atrophy in the CA1 subfield of the AD patients. How AD and DLB-typical pathologies compose the various local microenvironment of the hippocampus across AD and DLB needs to be further explored to understand this process. Additionally, microglia responses could further impact the atrophy rate. Some studies suggest that microglia react differently according to the underlying neurodegenerative disorder. How microglia are transformed across hippocampal subfields in AD and DLB, and how their changes are associated with disease-typical pathologies remains to be determined. To these purposes, we performed a volumetric analysis of phospho-Tau (P-Tau), Amyloid- β (A β), and phospho- α -Synuclein (P-Syn) loads, quantified and classified microglia according to distinct morphological phenotypes using high-resolution confocal 3D microscopy of hippocampal CA1, CA3 and DG/CA4 subfields of late-onset AD (n=10) and DLB (n=8) as well as age-matched control samples (n=11).

We found that each of the Tau, A β and Synuclein pathologies followed a specific subregional distribution, relatively preserved across AD and DLB. P-Tau, A β and P-Syn burdens were significantly exacerbated in AD, with Tau pathology being particularly severe in the AD CA1. P-Tau and P-Syn burdens were highly correlated across subfields and conditions ($R^2_{\text{Spear}} = 0.79$; $P < 0.001$) and result from a local co-distribution of P-Tau and P-Syn inclusions in neighbouring neurons, with only a low proportion of double-positive cells.

In parallel, we assessed the changes of the microglia responses by measuring 16 morphological features of more than 35,000 individual microglial cells and classifying them into seven-distinct morphological clusters. We found microglia features- and clusters-variations subfield- and condition-dependent. Two of the seven morphological clusters, with more amoeboid and less branched forms, were identified as disease-enriched and found to be further increased in AD. Interestingly, some microglial features or clusters were associated with one but more often with a combination of two pathologies in a subfield-dependent manner.

In conclusion, our study shows a multimodal association of the hippocampal microglia responses with the co-occurrence, distribution and severity of AD and DLB pathologies. In DLB hippocampi, pathological imprint and microglia responses follow AD trends but with lesser severity. Our study suggests that the increased pathological burdens of P-Tau and P-Syn and associated microglia alterations are involved in a more severe deterioration of the CA1 in AD as compared to DLB.

Introduction

The decline of memory is an overlapping symptom in age-related neurodegenerative diseases (NDDs)¹. The hippocampus is at the centre of numerous cognitive processes and is essential for spatial and episodic memory formation and consolidation²⁻⁴. It is one of the most severely affected brain regions in Alzheimer's disease (AD) and Dementia with Lewy Bodies (DLB)⁵⁻⁷ but the causes of its vulnerability are still poorly understood. The loss of hippocampal volume is associated with memory impairment in normal ageing⁸ and is predictive of AD and associated dementia⁹. Its architecture has been widely studied for its tri-synaptic loop circuitry and is composed of 5 subfields, the *dentate gyrus* (DG) as well as the *Cornu ammonis* (CA) fields CA1, CA2, CA3 and CA4¹⁰. Hippocampal subfields are distinct in their function and cellular composition and could be dedicated to or specialized in distinct types or sequences of memory processes¹¹⁻¹³. Interestingly, the atrophy of the hippocampus follows specific subregional patterns between AD and DLB cases, with the CA1 subfield particularly affected by neuronal loss in AD¹⁴⁻¹⁹. Understanding the mechanisms that lead to subregional hippocampal vulnerability and deterioration in AD and DLB could unveil personalized therapeutic strategies to alleviate the progression of dementia in NDDs.

At the cellular level many factors could be involved in the accelerated deterioration of CA1 in AD. NDDs are associated with extra or intra-cellular deposits of misfolded proteins across the CNS parenchyma that are correlated with disease progression. Extracellular senile plaques made of Amyloid- β (A β) peptides, intracellular neurofibrillary tangles (NFT) built from hyperphosphorylated Tau (P-Tau) and intracellular aggregation of phosphorylated α -Synuclein (P-Syn) such as Lewy bodies²⁰, are diagnostic neuropathological hallmarks in NDDs²¹⁻²⁴. The overlapping of Tau, A β and Synuclein pathologies is frequent in DLB but also in AD in approximately two-thirds of the cases²⁵⁻³². It raises numerous questions about how intertwined neurodegenerative mechanisms are across conditions and their impact on the progression of the disease. Microglia, the innate immune cells of the CNS, regulate homeostasis by clearing

pathogens, cell debris and dying cells in the brain parenchyma³³. In cell cultures and rodent models, Tau, A β and Synuclein pathologies are all three strongly associated with microglial activation and responses^{34–40}. If these interactions prime microglial cells to alleviate or exacerbate NDDs progression is still under discussion. Microglia aggregation is typically observed around A β plaques, where microglia have been involved in A β 1-42 peptide and oligomer clearance but also in the formation and maintenance of A β plaques^{41–45}. Microglia attached to A β plaques also display distinct molecular signatures^{40,46,47}. Their relationship with Tau pathology spreading is still under debate with contradictory results emerging from studies using different mouse models^{48–50}. Furthermore, recent studies report a potential role of microglia in P-Syn clearance⁵¹ and its cell-to-cell transfer⁵². Additionally, the identification by genome-wide association studies (GWAS) of risk loci associated with genes involved in their physiology and responses such as CR1 (coding for the Complement receptor type 1), SPI1 (Transcription factor PU.1), TREM2 (Triggering receptor expressed on myeloid cells 2) and CD33⁵³ emphasizes on the association of their functions with AD onset. Maladaptive responses such as uncontrolled phagocytic activities or oversecretion of pro-inflammatory factors such as the tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and reactive oxygen species (ROS), could accelerate neuronal deterioration⁴². Furthermore, reports of regional and/or temporal heterogeneity of microglia signatures across mouse models and neurodegenerative conditions in human increase the complexity to define how and when microglia play beneficial or detrimental roles^{54–57} and raise the importance to better define their changes in association with the mixed pathologies contexts of patient samples. Some studies have already highlighted differences in changes and activation in the microglia network between AD and DLB^{58–63} with the microglia in DLB samples seemingly less altered and responsive. However, how microglia responses are associated to AD- and DLB-pathology burdens in the human hippocampus is still an open question.

In this study, we investigated how the co-occurrence of Tau, A β and Synuclein pathologies modifies the subregional pathological burden and microglia morphological changes across the hippocampus of AD and DLB cases. We immunostained thick sections with P-Tau, A β , P-Syn or Iba1 antibodies and employed high-content 3D confocal microscopy combined with image analysis tools such as our Microglia and Immune Cells Morphologies Analyser and Classifier (MIC-MAC) pipeline on a collection of neuropathologically diagnosed AD and DLB cases, and age-matched controls (CTLs) samples. Our analysis revealed a specific distribution pattern across hippocampal subfields for each pathology, a strong correlation between P-Tau and P-

Syn loads, as well as a fine-tuned association between microglia morphological changes and co-occurrence and severity of AD- and DLB-pathologies across subregions and conditions.

Materials and methods

Human brain samples and processing

All experiments involving human tissues were conducted in accordance with the guidelines approved by the Ethics Board of the Douglas-Bell Brain Bank (Douglas Mental Health University Institute, Montréal, QC, Canada) and the Ethic Panel of the University of Luxembourg (ERP 16-037 and 21-009). All anonymized autopsy brain samples were obtained from the Douglas-Bell Brain Bank (see Table 1). Hippocampal samples (median part of the hippocampus, between the uncus and *corpus geniculatum laterale*) were dissected from cases of neuropathologically confirmed AD and DLB, as well as from age matched CTLs. Samples were evaluated regarding A β plaques, NFT and/or alpha-synuclein and assessed according to Braak and ABC staging ^{21,64} or Mc Keith staging criteria ⁶⁵ by a neuropathologist affiliated to the Douglas-Bell Brain Bank. This study comprises age-matched CTLs with no history of dementia. However, some CTL cases showed low level of neuropathological abnormalities such as AD pathology. Human brain samples from the Douglas-Bell brain bank used for the volumetric study were preserved in 10% formalin until processing as indicated in Table 1.

Post-mortem case description

The description of the samples is detailed in Table 1. We ran our image-based analysis on 11 age-matched CTLs, with no or low levels of neuropathological alterations as well as 10 AD (A1B2C2 to severe stages A2B3C2 or Braak IV) and 8 DLB (limbic transitional to neocortical diffuse) cases bearing often a certain amount of AD pathologies. The average year of death and post-mortem delay (PMD) did not differ significantly between groups and are 83.1 years and 22.56 hours for CTLs; 87.1 and 23.14 for AD; and 77.3 and 19.4 for DLB. Gender representation is slightly unbalanced with female cases being respectively 54.5% for CTLs, 30% for AD and 37.5% for DLB. Average time spent in fixative was also approximatively similar for specimens of all groups (CTLs, 14.7 years; AD, 11.1 and DLB, 12.25).

Immunohistochemistry

One sample from the Luxembourg Brain Bank was used to validate P-Syn antibodies on 3 μ m Formalin-fixed paraffin-embedded (FFPE) sections. Briefly, sections were processed by an

automated immunostainer (Omnis Immunostainer, Agilent, Glostrup, Denmark) with primary antibodies against PS129-synuclein. Heat retrieval was performed with the Envision Flex Tris low pH buffer (Agilent) 30 min at 97°C on board, primary antibodies were incubated 20 min at RT, and detection was performed with the Envision Flex Detection kit (Agilent) based on the DAB/HRP substrate system.

Before sectioning, human hippocampal brain samples from the Douglas-Bell Brain Bank preserved in fixative were washed in Phosphate Buffer Saline (PBS) and cryo-preserved in 30% sucrose in PBS for 36 h approximately. Human samples were embedded in M-1 embedding matrix (Thermo scientific, USA) and cut into 50 µm to 100 µm thick slices on a sliding freezing microtome (LeicaSM2010R) and preserved at -20°C in a cryoprotectant solution (Ethylene glycol (30%), and glycerol (30%) in 0.05 M phosphate buffer (PB, pH 7.4)) until processed for immunohistochemistry experiments. Immunostainings were performed as previously described^{66,67}. In brief, after overnight UV irradiation (UV lamp Ushio, 30 Watt), sections were permeabilized for 30 min with 0.5% Triton-X 100 in PBS 1X. Subsequently, free-floating sections were incubated for 2 h with blocking solution (0.5% Triton-X 100 and 2% horse serum in PBS 1X) at room temperature (RT), incubated with primary antibodies in blocking solution for 72 h at 4°C. Sections were then washed three times for 10 min in PBS 1X, and subsequently incubated in 0.5% triton-X 100/PBS 1X at RT for 2 h with fluorophore coupled secondary antibodies. Some slices were additionally incubated for 20 min with the fluorescent DNA dye DRAQ7TM (1:100, Cell Signaling #7406) at RT and washed twice for 10 min in 0.1 M PB (pH 7.4). Sections were finally washed twice for 10 min in 0.1 M PB (pH 7.4) prior to mounting on glass slides using ProLong Gold Antifade reagent (Invitrogen). All references for primary and secondary antibodies can be found in Table 2, respectively Table 3.

Image acquisition and analysis

3D image acquisition

Confocal images were captured on a Zeiss LSM 710 or LSM 800 confocal system with a 20x air objective. 3D tile scans (z-steps of 1µm) of human hippocampal subfields from age-matched CTLs, DLB and AD patients were stitched via Zeiss or Imaris Stitcher and visualized with Imaris 9.5 and 9.6 (Oxford Instrument). 3D stacks of Iba1 for microglia morphology and for P-Tau, Aβ and P-Syn inclusions were acquired on serial sections in the anatomically defined regions of interest representative of the entire layered structure of the CA1, CA3 and DG/CA4:

from *Stratum oriens* to *Stratum moleculare* for CA1 and CA3, and of the DG including the hilus (CA4) as indicated in Supplementary Fig. 1.

Volumetric quantification of P-Tau, A β and P-Syn pathologies

To improve homogeneity in the analysis, for each marker, samples were stained following the same combination of primary and secondary antibodies (respectively AT8 with anti-mouse Alexa Fluor 647, 4G8 with anti-mouse Alexa Fluor 488 and 11A5 with anti-mouse Alexa Fluor 488). 3D stacks were acquired following the same parameters for confocal laser scanning (magnification, resolution, laser power, offset and dwell time). All subfields were acquired from the same slide. All segmentations of the protein inclusions were done on Imaris 9.5.1 and 9.6 with the surface module following the same procedure for each marker (surface detail, absolute intensity, thresholds) (Fig. 1A). After segmentation, all volumes of the resulting objects (3D reconstructions) were summed up. The final score (%) represents the percentage of the volume of the stack covered by the selected marker staining.

MIC-MAC 2.0

Microglia and Immune Cells Morphologies Analyser and Classifier (MIC-MAC) 2.0 is a software that allows for automatic segmentation of microglia and immune cells from fluorescent microscopy 3D acquisition. With respect to the previous version⁶⁸, automation and time performance have been improved.

New Automated Segmentation of Microglia

The image processing pipeline is now fully automated. While the previous version was based on a semi-supervised approach, meaning that a portion of the acquisition was needed to be labelled as positive signal or background, the new version automatically segments structures of interest by tuning parameters related to the image processing pipeline. A flowchart of the image processing pipeline is presented in Supplementary Fig. 2. The algorithm works by elaborating the 2D images of the 3D stacks one by one. First, a noise filtering is applied⁶⁹ with $\sigma = 25$, followed by a background subtraction phase made with a gaussian filter ($\sigma = [10 - 50]$). After an intensity adjustment [matlab::imadjust], the image is deconvolved by using the Lucy-Richardson method^{70,71}. Finally, positive pixels are detected via global thresholding by defining a parameter for the threshold t . The only two parameters that have been changed throughout this phase are σ from the gaussian filter and t from the global thresholding. The parameters have been tuned accordingly to the amount of noise present in the image, which can change due to numerous factors (e.g. microscope parameterization, tissue

conservation and antibody penetration). All morphologies of microglia were further validated by overlaying the segmented microglia object on the original 3D stack with the Iba1 staining.

Feature extraction and selection

Automated morphological feature extraction is performed at cell resolution⁶⁸. Out of 62 morphological features and derived measures from the first version of MIC-MAC, we have selected only a subset of 16 features by discarding highly correlated features (Pearson, higher than 80%). Among the 16 kept features are morphological and graph-based measures, that all represent a certain aspect of the 3D morphology and help to measure fine changes and alterations. A detailed description for each of the 16 features can be found in Supplementary Fig. 3 with an illustration of prototypical minimum and maximum extreme values.

Artifact Removal

Segmentation artifacts were automatically removed in three different steps: (1) By thresholding cell volume and keeping cells whose volume is between $\sim 48,000$ and $\sim 216,000 \mu\text{m}^3$, similar to values already present in literature. The volumetric range was visually validated. (2) By considering the amount of cell body surface that is included in the border of the acquisition. This allows to remove cells whose shape is incomplete and thus may alter the results. We specifically remove cells whose shared surface with the border is bigger than 7.5% of the total cell surface. This threshold has been manually tuned by visual validation. (3) By training a classifier based on morphological features. We trained a classifier (Boosted Tree – Ada Boost)⁷² via manual labelling of artifacts and applied the classification to the whole dataset with k-fold validation to prevent overfitting. The removal via cell volume is performed right after the image processing segmentation in order to speed up the feature extraction phase. The latter two, are performed after the feature extraction phase right before the analysis.

Cluster analysis

All 16 selected features were initially normalized to the range 0 to 1 over the complete set of cells. We used Ward hierarchical clustering⁷³ on the set of 16 features. All the features have been normalized by range into a mapping from 0 to 1 renderings of cells belonging to specific clusters at different heights of the dendrogram. We assessed that $n=7$ was the best set-up to segregate distinct morphological clusters. These differences were further validated by the statistical analysis of features distribution among clusters. Cells were subsequently projected on a Uniform Manifold Approximation and Projection (UMAP)⁷⁴ for visualization purpose.

Statistical tests

For statistical comparisons between populations, we used Mann-Whitney U Test. For evaluating score and p-value of correlations we used Spearman Correlation. All tests were performed at a significance level of 5 %. In accordance with the explorative nature of the analysis, no correction for multiple testing was necessary.

Data availability

Source code and raw data will be made publicly available after the peer-revision process.

Results

Co-occurrence and severity patterns of Tau, A β and Synuclein pathologies in the hippocampal subfields of AD and DLB samples.

To characterize the differences between Tau, A β and Synuclein pathologies across AD, DLB and age-matched CTLs, we quantified the volumetric distribution of P-Tau, A β and P-Syn using high-resolution confocal microscopy. The analysis of the fluorescent staining of AT8 for P-Tau and 4G8 for A β antibodies, which are typically used for neuropathology assessment, both showed expected inclusions and labeled structures, i.e. typical paired helical filaments (PHF) and NFT with AT8⁷⁵, A β plaques and some amyloid precursor protein (APP) detected in neuronal soma with 4G8⁷⁶ (Fig. 1A-B; Supplementary Fig.1B). The 11A5 P-Syn staining revealed different types of inclusions, from Lewy neurite-like structures to intracellular granular staining (Fig. 1C; Supplementary Fig.1B). To validate the 11A5 antibody against PS129 α -Synuclein, we immunostained 3 μ m-thick serial FFPE sections of the amygdala from a Lewy body positive DLB case with 11A5, 81A (Millipore) and EP1536Y (Abcam) antibodies all directed against the same target PS129. All antibodies revealed a similar distribution pattern associated with Synuclein pathology such as Lewy neurites, Lewy bodies and intracellular vacuolar aggregations in neurons (Supplementary Fig. 4A). We then immunostained 100 μ m-thick sections of PFA-fixed subiculum-entorhinal cortex of the same case, with a combination of an anti-PS129 antibodies (11A5, 81A or EP1536Y), an antibody against pan-axonal neurofilament to detect neurons (NF-H) and DRAQ7TM to stain nuclei and analyzed the 3D confocal images (Supplementary Fig. 4B). Again, all stainings between PS129 antibodies were broadly similar with labeled structures resembling Lewy bodies and Lewy neurites found enclosed in neurons double-stained for NF-H and DRAQ7TM. The 11A5 positive structures

found in our cohort of AD and DLB samples, also resembled Lewy neurites and Lewy bodies but also vacuolar aggregations and PHF-like, which were also seen with the 81A antibody (Supplementary Fig. 5).

After validation of the 11A5 antibody, we acquired representative 3D image stacks of AT8, 4G8, or 11A5 staining of the CA1, CA3 and DG/CA4 subfields for each case. The images were segmented with Imaris software 9.6 to estimate the relative volume covered by the pathology of interest (Fig. 1 A-C). We found variations in the pathological protein loads across samples and conditions that were generally compatible with the neuropathology staging of the samples. However, we observed all three P-Tau, A β , and P-Syn pathologies in AD as well as in DLB cases. The majority of DLB cases were already described with partial AD pathologies. The age-matched CTLs showed no or low level of P-Tau staining and low to moderate A β labelling in neuronal-like soma and in few extra-cellular deposits. In our CTLs group, we additionally observed very few P-Syn intra-cellular inclusions in cells looking like pyramidal neurons but no Lewy bodies or Lewy neurite like structures. The AD group typically showed the highest levels for all pathologies in the three hippocampal subfields. The DLB group showed low to intermediate levels of P-Tau, A β , and P-Syn accumulations (Fig. 1 D-F).

More precisely, P-Tau burdens were significantly higher in all subfields in AD and DLB compared to the CTLs (AD vs CTLs: $P < 0.001$, DLB vs CTLs: $P < 0.05$) and followed a similar subregional pattern across conditions with the highest loads found in CA1 and CA3 and lowest in DG/CA4. The severity of the Tau pathology was considerably higher with the most extreme values in CA1 AD, and DG/CA4 AD compared to DLB respective subfields ($P < 0.001$ in CA1; $P < 0.05$ in DG/CA4). We found a distinct subregional pattern of A β loads, again, conserved across the conditions and CTLs with the highest A β burdens in DG/CA4 and CA3 and lowest in CA1. Only A β AD values were significantly higher than CTLs ones for all subfields ($P < 0.05$). The subregional distribution of P-Syn loads appeared more uniform across subfields but with the highest loads found in CA1 and CA3 in AD and DLB. To be noted, the volume recovered by P-Syn staining were very low compared to A β or P-Tau ones. We found statistical significances for all subfields compared to CTLs for AD ($P < 0.001$), and for CA1 ($P < 0.05$) and CA3 ($P < 0.01$) in DLB. Surprisingly, the P-Syn burdens were much higher in AD than DLB samples for all subfields ($P < 0.05$ CA1, $P < 0.001$ CA3 and DG/CA4). Our data suggest that Tau, A β and Synuclein pathologies follow a specific subregional hippocampal pattern that is shared across AD and DLB. However, burdens were more severe in AD, with CA1 AD particularly affected by P-Tau accumulations.

Next, we examined the association between the three pathologies across subfields and conditions. Positive associations between P-Tau and A β and between P-Syn and A β volumes of respectively $R^2_{\text{Spear}} = 0.30$ and 0.26 were found across subfields and conditions but differ subregionally with superior values in the CA1 (respectively $R^2_{\text{Spear}} = 0.42$ and 0.37 ($P < 0.05$)) and a loss of statistical significance in the CA3 and DG/CA4. However, we found a strong positive correlation between P-Tau and P-Syn volumes across conditions ($R^2_{\text{Spear}} = 0.79$; $P < 0.001$) that was preserved in all subfields (CA1: $R^2_{\text{Spear}} = 0.75$; CA3: $R^2_{\text{Spear}} = 0.79$; DG/CA4: $R^2_{\text{Spear}} = 0.78$; $P < 0.001$) (Fig. 2A-B). To better define P-Tau and P-Syn associations at the cellular level, we tested double staining of P-Tau with a P-Ser422 rabbit antibody to pair with P-Syn (11A5, mouse) and DRAQ7TM (Fig. 2C). We confirmed that P-Tau P-Ser422 (rabbit) staining colocalized with AT8 ones in a CA1 AD case (Fig. 2D). We observed a global co-distribution of P-Tau and P-Syn in the same microenvironment, however, colocalizations in the same neuron were sparse and mono-stained neurons more common. Thus, the strong positive correlation between P-Tau and P-Syn does not point out intertwined intracellular co-pathologies but rather simultaneous mono P-Tau or P-Syn accumulations in neighboring hippocampal neurons.

Microglia morphological responses in hippocampal subfields of AD and DLB patients

To assess the subregional microglia responses that paralleled the local pathological context, we took advantage of a pipeline for image analysis, previously published, that allows for semi-automated segmentation and classification of microglia cells based on their morphologies⁶⁸. Indeed, morphologies of microglia partially reflect their responses; surveying microglia are usually very ramified, whereas activated microglia present an amoeboid-like morphology. For the purpose of this study that requires a time-consuming processing, we first implemented to our microglia morphological classifier called Microglia and Immune Cells Morphologies Analyser and Classifier (MIC-MAC)⁶⁸ a 2.0 version which offers numerous improvements. MIC-MAC 2.0 allows a fully automated, more precise and faster segmentation of large 3D confocal stacks, an automated discard of the stack-border microglia that could create artifact structures and a simplified analysis relying on 16 morphological features that recapitulate in detail the core parameters of microglia 3D morphology (Supplementary Fig. 2 and 3). For this study, we captured more than 35,000 individual microglia across 87 3D stacks, representative of each of the three hippocampal subfields for each case of our cohort. Before extraction of the 3D features of microglia, we found a comparable density of Iba1 staining across subfields and

conditions (Supplementary Fig. 6). After segmentation, MIC-MAC 2.0 imposed a volume threshold to select individual microglia but discard microglia accumulations that appeared as merged structures, such as the plaque associated microglia, from the analysis.

Variations of microglia morphological features across hippocampal subfields in AD and DLB cases

First, we measured the 16 morphological features of extracted microglia (list and definition in Supplementary Fig. 3) to assess the precise changes in their morphologies across subfields and conditions. Interestingly, we found that DLB values present a trend similar to AD samples for almost all features and subfields, but without reaching significance. Among the 16 features, we found only four of them significantly different in AD compared to CTLs in at least one subfield. The compactness was the only feature significantly increased in all subfields in AD compared to CTL ($P < 0.05$ for CA1 and CA3, $P < 0.001$ for DG/CA4) (Fig. 3A). Volume/number of edges significantly increased, and node density decreased in CA1 (both $P < 0.05$) and DG AD (respectively $P < 0.05$ and $P < 0.01$) (Fig. 3B-C). Ending nodes density increased in CA3 AD ($P < 0.05$) (Fig. 3D). Intra-condition, we found that the feature average node degree is significantly higher in CA1 AD than in CA3 and DG/CA4 AD ($P < 0.05$ and $P < 0.05$; Fig. 3E), S-metric and node density lower in CA3 than DG in DLB ($P < 0.05$ Fig. 3F, and $P < 0.05$ Fig. 3C). Overall, compared to the CTLs microglia from the same subfield, CA1 AD microglia adopted a general morphology with higher compactness and loss of branching. AD DG/CA4 microglia followed a similar trend but to a lesser degree. AD CA3 microglia also showed a decrease in their complexity but with a distinct fine-tuning of their morphology.

Microglia morphological clusters composition in the hippocampus AD and DLB patients.

Features analysis allowed us to dissect the fine details of microglia morphological changes but does not reflect on how microglia populations are composed at a local level. To further characterize how microglia populations are remodeled across the hippocampal subfields in AD and DLB, we applied a cluster analysis to our matrix of morphological data. Briefly, we have selected seven clusters after a Ward hierarchical clustering based on a normalized set of the 16 features (Fig. 4A). The seven clusters show distinct morphological patterns and specific characteristics and were distributed accordingly on an UMAP with prototypical morphologies (Fig. 4B and Supplementary Fig. 7A). Briefly, clusters 1, 2 and 7 are composed by branched cells with high node density, volume and polarity. Cluster 3 represents the most compact and smallest microglia. Clusters 4, 5 and 6 show intermediate shapes mainly segregated by distinct

features such as volume (higher in cluster 5), mean edge length (higher in cluster 4, not shown), and node density (higher in cluster 6) (Supplementary Fig. 7B-G). We then analyzed clusters relative abundances in CTLs, AD, and DLB. We found a general decrease of the most ramified clusters 1 and more severely of cluster 7 in AD compared to CTLs (respectively $P < 0.05$ and $P < 0.001$) and in DLB for 7 ($P < 0.05$). Cluster 3 and 5 were the two clusters enriched in both disease conditions (Fig. 5A) (cluster 3: AD $P < 0.001$, DLB $P < 0.01$) (cluster 5: AD $P < 0.001$, DLB $P < 0.01$). Clusters 2 and 6 showed similar ratios in all conditions and CTLs but cluster 4 was slightly but significantly increased in AD vs CTLs ($P < 0.05$) (Fig. 4C). At the subregional level, all conditions taken together, we found cluster 4 in lower quantity in DG/CA4 compared to CA3 ($P < 0.01$) (Supplementary Fig. 8). When we analyzed the changes of cluster composition by subfield across conditions, we reached significance for cluster 3 increase for all subfields in AD compared to CTLs ($P < 0.05$ in CA1; $P < 0.01$ in CA3 and $P < 0.05$ in DG/CA4) and in CA3 DLB ($P < 0.05$); for cluster 5 increase in CA1 AD, DG/CA4 AD ($P < 0.01$) and CA3 DLB ($P < 0.05$), and for cluster 7 decrease in CA1 and DG/CA4 AD ($P < 0.05$) (Fig. 4D). Overall, we observe a tight regulation of the morphological cluster composition across subfields, with an enrichment of the two potential “disease” subgroups in AD, especially in the CA1 and DG/CA4, and a depletion of the ramified cluster 7. In DLB, alterations appeared more profound in the CA3 with the cluster 5 increase balancing cluster 7 decrease. The stability of cluster 2 and 6 across conditions imply that some subgroups of microglia do not react to the abnormal protein accumulations. The morphological cluster composition highlights the microglia functional heterogeneity. Some clusters specifically changed depending on the subfield and condition, and others were seemingly unreactive to the pathological context. This analysis further implies a subregional influence on microglia at the hippocampus level. In the next step, we investigated how these subfield specificities could tie up to the pathological context.

Associations between morphological microglia changes and P-Tau, A β and P-Syn loads in AD and DLB hippocampi

To further estimate how each pathology could modulate microglia responses in the hippocampus, we first evaluated the associations (statistically significant) between morphological features and P-Tau, A β and P-Syn burdens across subfields (Fig 5). Interestingly, the associations of P-Tau, A β and P-Syn with features changed across subfields. In the CA1, we found significant correlations of some of the most altered features in AD with

P-Tau (4) and/or P-Syn (4), most of the time in combinations at similar correlation values. In the CA3, some features were significantly associated with P-Tau (4) in pairs with A β (2) or P-Syn (2), and 3 features uniquely to A β . In DG/CA4, a higher range of features were positively or negatively associated with A β (8), sometimes in pairs with P-Tau (3), with P-Tau alone (1) and P-Tau in pairs with P-Syn (1). The compactness, a feature statistically increased in AD, was associated with P-Tau loads in all subfields, but in pair with A β only in the CA3 and DG/CA4. Volume/number of edges (Nedges) also changed in AD CA1 and DG/CA4 showed an association with both P-Tau and P-Syn in the CA1, and with P-Tau and A β in DG/CA4. This data implies that P-Tau in association with P-Syn pathology triggered the most significant changes in the CA1, however A β when highly expressed is a more dynamic modifier of microglia morphology.

At the cluster level, we also found a strong effect of the subregion on the associations between pathologies and clusters (Fig 6). First, we observed similar intercluster associations across the subfield. The increased “disease” cluster in AD and DLB, 3 and 5, are the most strongly positively associated in CA1 with this association preserved in CA3 and DG/CA4. Cluster 3 maintains a negative correlation with cluster 1 and 7 in all subfields. Cluster 5 stays positively correlated with cluster 4 in all subfields and negatively associated with cluster 7 in all subfields. Cluster 7, the largest subgroup in CTLs, seems to be depleted in favor of clusters 3 and 5, with cluster 3 potentially partially resulting of cluster 1 transformation. Cluster 5, poorly ramified, could also represent a transitional form toward cluster 3, more amoeboid-like, but stays in higher proportion across disease and subfields. Again, the stability of cluster 2 and 6 may indicate non-reactive microglia subgroups to their pathological micro-environment. Then, we found that cluster relationship with P-Tau, A β and P-Syn burdens varied among subfields. In CA1, we found positive correlations between cluster 5, and negative ones for cluster 7, and the pair of P-Tau and P-Syn loads, and positive one between cluster 4 and P-Syn. In CA3, cluster 3 was positively correlated with the pair of P-Tau and A β loads, clusters 4 and 5 with P-Tau loads. In DG/CA4, cluster 3 was associated with A β loads only and cluster 4 with P-Tau ones only, cluster 5 and 7 with the pair of P-Tau and A β loads.

The strong associations between P-Tau, A β and P-Syn pathologies and microglia morphologies suggest a tight inter-dependence of the pathological context and microglia responses. The severity, distribution but also co-occurrence of the three pathologies could impact microglia phenotypic heterogeneity and condition the level of their response.

Discussion

Here we studied the subregional distribution and severity of the P-Tau, A β and P-Syn pathologies along with the morphological changes of microglia in the hippocampal CA1, CA3 and DG/CA4 subfields across age-matched CTL, AD and DLB conditions. We have obtained high-content data about subregional patterning of pathologies in the human hippocampus across conditions and its relationship with the remodelling of the microglia local population.

P-Tau, A β and P-Syn burdens overlap and show specific subregional patterns in the hippocampus of AD and DLB cases.

Here we report a high co-occurrence of the three types of pathologies studied, Tau, A β , and Synuclein in hippocampal subfields of AD and DLB cases. P-Tau, A β and P-Syn burdens were all more severe in AD. The concomitance of AD- and DLB-pathologies is commonly observed in age-related NDDs⁷⁷ and reported in both AD and DLB^{24,27} in numerous brain areas^{26,31,32,78}. However, our study revealed a complex tapestry of subregional pathological imprint in AD and DLB hippocampi, which each of the pathologies showing a specific subfield pattern.

We found that P-Tau loads were more enriched toward CA1 and A β ones toward DG/CA4. P-Syn loads were slightly higher in CA1/CA3 but appeared more homogeneous across subfields. DLB samples presented intermediate values of AD-typical and Synuclein pathologies. In this study, we have analyzed the medium part of the hippocampus, i.e., the body, between the uncus and *Corpus geniculatum laterale*, a region which is usually poorly described, contrary to the head at the uncus level or the tail close to the *Corpus geniculatum laterale*, both more regularly used for neuropathology diagnostic and research. We cannot exclude differences with previous neuropathological reports as it has been shown that the hippocampus is molecularly and functionally diverse along its longitudinal axis^{79,80} and may be affected by different local level of pathologies. Finally, our quantification method, based on 3D confocal images, is more precise and reflects volumetric measures compared to the traditional surface measures. Our data are generally consistent with existing literature regarding the subregional distribution of Tau pathology in AD and DLB^{81,82}, but slightly differ regarding A β subregional distribution⁸³ and P-Syn higher loads in AD. We also report low A β and P-Syn accumulations but almost no P-Tau inclusions in our age-matched non-demented CTLs, which is in accordance with previous reports^{84–89}. Even though the P-Syn burdens that we have measured represent very low volumes compared to A β and P-Tau ones, the use of high-resolution confocal microscopy allowed us to reveal frequent P-Syn intracellular granular inclusions in cells identified as

pyramidal neurons. The higher P-Syn loads in AD cases compared to DLB seem at first counter-intuitive. We think that the hippocampus is relatively preserved of a more spread and severe Synuclein pathology as we have observed only a few Lewy body-like structures in our analysis. The typical accumulations of P-Syn into granules that we observed in cells identified as pyramidal neurons could represent an early or light stage of Synuclein pathology in the hippocampus. The few P-Syn granules found in CTLs samples could be in favor of such an hypothesis.

Interestingly, we reported stronger associations between P-Tau and P-Syn burdens than between P-Tau or P-Syn and A β loads. By high-resolution confocal microscopy, we found that the positive correlation between Tau and Synuclein pathologies depended mainly on exclusive distribution of P-Tau or P-Syn inclusions in neighbouring hippocampal neuronal populations rather than on a colocalization in the same neuron that could suggest a systematic mechanistic association. However, we observed rare P-Tau and P-Syn double-positive hippocampal neurons carrying the two types of pathologies such as previously found in the subiculum⁹⁰, the amygdala⁹¹ and parahippocampal gyri⁹² in AD and DLB cases. In a previous study, the local co-distribution of NFTs and Lewy bodies was also found more frequent in limbic areas than other brain parts²⁶. How P-Syn- and P-Tau-bearing neighbouring neurons co-inhabit the AD and DLB hippocampus and how the two pathologies impact each other remains to be understood.

Hippocampal microglia morphological remodelling is impacted by complex subregional pathological context.

Morphology of microglia only partially reflect their responses. However, visual description is commonly used to obtain a rapid readout of their alterations in CNS diseases. Indeed, the homeostatic microglia harbor a highly ramified profile in contrast to activated microglia that appear smaller, amoeboid-like with no or low branches. Here, we have analyzed 3D morphologies to characterize the local reorganization of the microglia population in the hippocampus subregions of AD and DLB patients. We previously developed an AI pipeline called MIC-MAC, to classify and quantify morphologies of individual microglia labelled with the Iba1 marker in large 3D confocal image stacks of human or mouse postmortem brains⁶⁸. With MIC-MAC 2.0 presented here, we have significantly improved the automation and precision of the segmentation and added new features to discard artefacts upstream from the analysis. A caveat of our approach is that MIC-MAC 2.0 automatically discards all microglia accumulations, where the cells are so closely intermingled that our pipeline cannot separate

them into individual cells after the segmentation process. In our hands, even super-resolution microscopy could not resolve the human microglia cellular boundaries in these accumulations. This argument strengthened our decision to focus the MIC-MAC analysis on individual cells to overcome potential contaminations in our data. Our analysis created a substantial amount of information with a sampling of 35,000 extracted microglia. By quantifying their 16 morphological features, our analysis highlighted conditions and subregional particularities. When compared to their respective subfield in CTLs, we found statistical significances only for the AD condition. However, DLB variation trends always mimic AD ones showing that the presence of concomitant proteinopathies already initiated local remodeling even if subtler. Many reports found low to mild microglial changes in DLB samples. These conclusions were supported by 2D morphology analyses and quantifications of some activation markers in the hippocampus⁶¹ and other brain areas such temporal lobe, the superior frontal gyrus⁵⁹ and cerebral cortex⁶⁰. However, our analysis implies that DLB microglia are not so different from AD ones in their ability to respond and that their alterations are associated with the level of severity of DLB and AD pathologies in the parenchyma. High compactness was the only predictive feature for AD across subfields, strengthening the observation that microglia tend to become more amoeboid in AD, even when not attached to the plaques. At the morphological clusters level, as in previous reports^{61,93}, we show a decrease of the most branched and tortuous subgroups and an increase of amoeboid-like clusters significantly in AD. Interestingly the disease-increased clusters are also found at low levels in CTLs and could be products of the ageing process or the onset of pathological mechanisms⁹⁴. Our data also further suggest functional heterogeneity among microglia local subgroups. Indeed, we found that two morphological clusters stayed relatively stable over the conditions, while others were specifically depleted toward simplified morphologies. We have also uncovered two stereotypical morphologies enriched in AD and DLB, one amoeboid-like and one semi-ramified. It could reflect differences in response to pathological stressors and further illustrates the residual heterogeneity within the microglia population in health and disease⁵⁵. Our study demonstrates that Tau, A β and Synuclein pathologies impact the local microglia remodelling in the AD and DLB hippocampus. We found fine-tuned associations with specific features or clusters that reflect the tight interdependence of microglia states with its pathological environment. Microglia responses are often characterized in monogenic models where only one pathology is expressed, with these signatures often interpolated to the human condition. Many of the microglia AD signatures, such as the disease-associated microglia^{95–97}, have been characterized in monogenic animal models mimicking A β pathology and were often observed

in microglia surrounding A β plaques. If these signatures are fully or partially, regionally or sub-regionally recapitulated in human conditions still needs to be further characterized^{98–103}. Recently, Gerrits et al.¹⁰⁴ have defined by single-nuclei RNA sequencing that some signatures of microglia were selectively associated with A β , or with combined A β and Tau pathologies, in the occipital and occipitotemporal cortices of AD patients. Such molecular heterogeneity of microglia is expected in the AD hippocampus. Indeed, we observed that hippocampal microglia alterations are most often associated with dual pathologies. Microglia local responses are tightly correlated with the co-occurrence and severity of Tau, A β and Synuclein pathologies. It does not contradict our observation of the strong impact of A β on microglia morphologies in the CA3, which has also been described in amyloidosis mouse models. Nevertheless, it emphasizes the dual role of Tau and Synuclein pathologies on exacerbating microglia responses, particularly in the AD CA1. The complex pathological pattern of the human condition certainly represents a factor of divergence of microglia phenotypes between AD patients and amyloidosis rodent models.

Conclusion

Our analysis suggests that the co-occurrence of the three Tau, A β and Synuclein pathologies is frequent in the hippocampus of AD and DLB patients with a general severity largely increased in AD cases. Each pathology seems to follow a distinct subregional pattern, mainly conserved across AD and DLB. Our study also implies a synergistic effect of the three pathologies on microglia transformations that tightly regulate the morphological composition of the local microglia population. The combination of high burdens of P-Tau and P-Syn with increased microglia alterations could participate in accelerated atrophy of the CA1 in AD.

Acknowledgements

The authors thank the Douglas-Bell Brain Bank and its staff for providing human brain samples, the Bioimaging Facility of the Luxembourg Centre for Systems Biomedicine (LCSB) for support of microscopy, and the Reproducible Research Results (R3) team of the LCSB for promoting reproducible research. The 11A5 antibody was kindly provided by Dr. Manuel Buttini and Dr. Wagner Zago (Prothena Biosciences).

Funding

This work was supported by the Espoir-en-tête Rotary International awards, the Auguste et Simone Prévot foundation and the Agaajani family donation for Alzheimer's Disease research (to D.S.B.) and the Luxembourg National Research Fond (FNR: PEARL P16/BM/11192868 grant) (to M.M). S.F. and C.A. were supported by the PRIDE program of the Luxembourg National Research Found through the grants PRIDE17/12244779/PARK-QC and PRIDE17/12252781/DRIVEN respectively.

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available online.

Authors contributions

DS.B conceived and supervised the study. S.F produced IHC on fixed tissue, S.F and DS.B performed confocal microscopy acquisitions and volumetric analysis. C.A designed MIC-MAC 2.0, with inputs from S.F, L.S and DS.B for validations. C.A and G.H produced statistical data analysis. O.U.H, C.S and JJ.G produced some support experiments. N.M provided human fixed samples. M.M provided FFPE samples and resources. A.S provided bioinformatic resources. S.F, C.A and G.H produced figures. S.F, C.A, M.M and DS.B wrote the manuscript. All authors contributed to the final version of the manuscript.

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Figure legends

Figure 1. P-Tau, A β and P-Syn loads show distinct disease-associated, subregional patterns in the hippocampus of AD and DLB patients. P-Tau, A β , and P-Syn burdens were assessed via immunofluorescence staining (big panels and zoom in small upper panels) and volumetric analysis (volume percentage of 3D z-stack acquisition) in CA1, CA3 and DG/CA4 of age-matched CTLs, AD and DLB-mixed AD patients. The staining signal was segmented (with Imaris 9.6) (small lower panels) and their volume measured from 3D high-resolution acquisitions of each hippocampal subfield for each sample. (A) Paired helical filaments and neurofibrillary tangles of P-Tau (cyan) were stained with anti-AT8, example of an 80-year-old male AD patient (case 21). (B) A β plaques and subcellular inclusions (magenta) were stained with anti-4G8, example of a 90-year-old male AD patient (case 30). (C) P-Syn inclusions, Lewy bodies and Lewy neurites (green) were stained with anti-11A5, example of a 64-year-old male DLB patient (case 15). Violin plots (with box plots) show that (D) P-Tau levels are significantly higher in the AD samples as compared to both DLB and CTLs in all three hippocampal subfields, but a significant increase was also observed in DLB samples compared to age-matched CTLs. (E) A β levels are significantly higher in AD samples in all three subfields compared to CTLs, whereas no significant differences are seen between DLB and

both AD and CTL. A β loads follow the same subregional pattern in DLB but with lower values. (F) P-Syn loads are significantly increased in AD in all subfields, and in CA1 and CA3 in DLB compared to age-matched controls. P-Syn volumes are also significantly higher in AD samples compared to DLB. Wilcoxon-Mann-Whitney U-test p-values are indicated in the graphs: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$. Scale bars in A, B and C big panels = 200 μm , small panels = 30 μm .

Figure 2. P-Tau and P-Synuclein loads are strongly correlated across hippocampal subregions and conditions. The Spearman correlation bubble charts (log₂ scale) display a strong positive correlation between P-Tau and P-Syn loads across (A) conditions and subfields ($R^2_{\text{Spear}} = 0.79$) and (B) at subregional level: CA1 ($R^2_{\text{Spear}} = 0.75$), CA3 ($R^2_{\text{Spear}} = 0.79$) and DG/CA4 ($R^2_{\text{Spear}} = 0.78$). Corresponding A β loads are labeled by the size of the bubble. Lower but positive correlation are observed between P-Tau and A β ($R^2_{\text{Spear}} = 0.3$) and between P-Syn and A β ($R^2_{\text{Spear}} = 0.26$) across the three subregions. (C) The correlation between P-Tau and P-Syn is also visible at cellular level with mainly mono-positive neurons for one or the other type of inclusion, but also some rare double-positive neurons that contain P-Tau and P-Syn inclusions at the same time. CA1 from an 82-year-old male AD patient (case 22) immunostained for P-Syn (mouse, 11A5, green), P-Tau (rabbit, PSer422, Thermofisher 44-764G, magenta) and fluorescent DNA dye DRAQ7TM (blue). Rare pyramidal neurons that co-express P-Syn and P-Tau are indicated by full arrowheads. More commonly, pyramidal neurons are positive for either P-Tau (full triangles) or P-Syn (empty triangles) inclusions. To facilitate visualization of mono- respectively double-positive neurons, the location of arrows and triangles is kept in all staining images. Nuclei of all cells are stained in blue. (D) Colocalization (full arrows) of AT8 (mouse, cyan) and PSer422 (rabbit, magenta) is confirmed in the CA1 of a 90-year-old AD patient (case 28). Scale bars (C, D): 30 μm .

Figure 3. Subregional morphological alterations of microglia in the hippocampus of AD patients. Using MIC-MAC 2.0, we have extracted 16 geometrical features per cell of more than 35,000 individual microglia collected in CA1, CA3 and DG/CA4 subfields from AD, DLB and age-matched controls. None of the analyzed microglia morphological features was significantly changed in the DLB samples but follow a trend similar to the AD condition. All the following reported changes are observed in AD vs CTL samples. (A) Compactness is significantly increased in all three hippocampal subregions, (B) and volume over number of edges is significantly increased in CA1 and DG/CA4. (C) Node density significantly decreases

in CA1 and DG/CA4, as does ending nodes density in CA3 (D). Two features, namely average node degree and s-metric present significant intra-condition regional changes. Wilcoxon-Mann-Whitney U-test p-values are indicated in the graphs: $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$. Scaled prototypic morphologies with highest and lowest value for the selected feature have been added on the left side. Scale bar = 20 μ m.

Figure 4. Microglia morphological cluster distribution varies according to the disease and the subfield. Ward hierarchical clustering on the matrix of 35,000 microglia based on 16 features defines seven distinct morphological clusters. (A) Hierarchical clustering dendrogram. (B) Scaled prototypical morphologies of microglia associated to the seven clusters inside the UMAP. (C-D) Histograms showing the variation in abundance (average composition in %) of the seven clusters according to the condition (C) and across subfield per condition (D). Overall, clusters 3 and 5 are enriched in the disease conditions, whereas cluster 7 is depleted. Scale bar in B = 30 μ m. Wilcoxon-Mann-Whitney U-test p-values are indicated in the graphs: $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$.

Figure 5. Microglial morphological features correlate with P-Tau, A β , and P-Syn burdens in the hippocampus and follow a subregional pattern.

Correlation plots between microglial morphological features and pathologies in CA1 (A) and CA3 (B), and DG/CA4 (C) in CTL, AD and DLB conditions. A list and description of all morphological features with prototypic morphologies for lowest and highest value can be found in S.Fig 5. Various microglia morphological changes are correlated to one or two proteinopathies in a subregion-dependent manner. Statistically significant values (Spearman's correlation) are indicated by a dot, which color gives an estimation of the correlation (positive correlations in blue, negative correlations in red). Black crosses indicate insignificant correlations.

Figure 6. Microglial morphological clusters correlate with P-Tau, A β , and P-Syn burdens in the hippocampus and follow a subregional pattern.

Correlation plots between microglial morphological clusters and pathologies in CA1 (A) and CA3 (B), and DG/CA4 (C) of all conditions. In a subfield-dependent manner, disease-enriched clusters 3 and 5 are positively correlated with one or more pathologies whereas disease-depleted cluster 7 present negative correlations with proteinopathies. Statistically significant values (Spearman's correlation) are indicated by a dot, which color gives an estimation of the

correlation (positive correlations in blue, negative correlations in red). Black crosses indicate insignificant correlations.

Supplementary Figure 1. Hippocampal subfields and confocal stainings of P-Tau, A β and P-Syn across conditions and hippocampal subfields.

(A) Anatomically defined CA1, CA3 and DG/CA4 subfields of the hippocampus for proteinopathy and microglia 3D z-stack acquisitions. Hippocampal section of an 83-year-old female AD patient (case 24) with A β (4G8, green) and microglia (Iba1, magenta) stainings. (B) 50-100 μ m thick hippocampal sections were immunostained with the AT8 antibody against P-Tau (Ser202, Thr205) (cyan), the 4G8 antibody against A β (AA17-24) (magenta) and the 11A5 antibody against P-Syn (Ser129) (green). The stainings show heterogenous distribution and types of inclusions across conditions and hippocampal subregions. Scale bars in (A) = 1000 μ m, in (B) = 100 μ m.

Supplementary Figure 2 MIC-MAC 2.0 analysis workflow.

MIC-MAC 2.0 pipeline with illustrations of acquisition (A), automated image processing (B), manual validation via overlay (C), feature extraction (D), feature analysis (E) and cluster analysis (F). (A-C) Original Iba1 staining of microglia is represented in white, segmented microglia cells in magenta. (D) Microglia 3D geometrical features are based on nodes (connection points) and edges (branches). (F) Spatial cluster color coded overlay. Scale bars in (A-C), (F) = 100 μ m.

Supplementary Figure 3. Morphological features with minimum and maximum values.

List and description of all morphological features with prototypic morphologies for lowest and highest feature value. Significant changes based on Wilcoxon-Mann-Whitney U-test observed in AD samples are indicated with one ($P < 0.05$) or two arrows ($P < 0.01$) on the right side. Scale bars = 20 μ m.

Supplementary Figure 4. Validation of P-Syn stainings with three different antibodies on FFPE samples of a neuropathologically confirmed DLB case.

3- μ m thick paraffin block sections from the amygdala and 50-100 μ m thick sections from fixed hippocampus were obtained from the same 91-year-old male DLB patient (case 20) and were stained against P-Syn using three different antibodies, namely 11A5, 81A and ab51253, that all three recognize the P-Ser-129 epitope. (A) Sections from paraffin blocks were stained

against P-Syn (11A4, 81A and ab51253, brown) based on the DAB/HRP substrate system. The lower rows represent a zoom of the indicated region in the upper row. (B) Sections from fixed samples were stained by immunofluorescence against P-Syn (11A4, 81A and ab51253; green), neurofilaments (NF-H, magenta) and all nuclei (DRAQ7TM, blue). Scale bars in (A) upper row = 500 μ m and lower row = 50 μ m; (B) upper row = 100 μ m and lower row = 10 μ m.

Supplementary Figure 5. Confocal description of P-Syn staining in a neuropathologically confirmed DLB case.

50-100 μ m thick sections from fixed hippocampus from a 90-year-old male AD patient (case 28) were immunostained against P-Syn (11A5, 81A; green), neurofilaments (NF-H, magenta) and all nuclei (DRAQ7, blue). The upper row shows the hippocampus (*stratum oriens* at left bottom corner) at 20x magnification, and the lower row represents a zoom of the marked areas in the pyramidal layer. P-Syn inclusions are present under various forms, including PHF-like (full triangle), Lewy neurite (empty triangle) and vacuolar aggregations (arrowhead). Scale bars upper row = 50 μ m and lower row = 20 μ m.

Supplementary Figure 6. Iba1 density in hippocampal subfields of AD and DLB do not differ from age-matched CTLs.

Iba1 volume (%) across conditions and hippocampal subfields.

Supplementary Figure 7. Microglia morphologies can be divided in seven clusters.

(A) Projection on a UMAP of the seven clusters where each of the more than 35,000 individual microglia is represented by a dot. (B-D) Distribution spectrum for volume, compactness and polarity on UMAP. (E-G) Violin plots showing the enrichment on volume, compactness and polarity in specific clusters.

Supplementary Figure 8. Cluster distribution varies among hippocampal subregions.

Histogram showing the variation in abundance (average composition in %) of the seven clusters according to CA1, CA3 and DG/CA4 subregions. Only cluster 4 presents a significant change: a decrease in DG/CA4 compared to CA3. Wilcoxon-Mann-Whitney U-test p-values are indicated in the graphs: * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

Table 1. Demographic and neuropathological data of human samples.

Table 2. Primary antibodies.

Table 3. Secondary antibodies.

Table 1 Demographic and neuropathologic data of human samples

Diagnosis	Case	Gender	Age (years)	PMI (hours)	Disease Score	Reported concomitant Proteinopathies*
CTL	1	F	86	5.75	/	/
	2	F	80	17.5	/	/
	3	F	89	23.58	/	Some senile plaques and NFTs
	4	M	89	32.25	/	/
	5	M	80	13	/	Some senile plaques
	6	F	83	35.75	/	/
	7	F	95	23.75	/	/
	8	M	85	26.75	/	/
	9	M	83	16.83	/	/
	10	M	89	31.98	/	AIBICI
	11	F	55	21.07	/	/
DLB	12	M	70	24.5	Neo-cortical diffuse	Senile plaques and NFTs
	13	M	89	17	Limbic transitional	Mixed AD
	14	M	75	21	Neo-cortical	Senile plaques and NFTs
	15	M	64	17.25	Neo-cortical	Senile plaques and NFTs
	16	F	83	17.75	Neo-cortical	Senile plaques and NFTs
	17	M	82	22	Neo-cortical diffuse	/
	18	F	81	8.58	Neo-cortical diffuse	/
	19	F	74	27.62	Neo-cortical	A2B2C0
	20	M	91	85	Neo-cortical diffuse	Braak III/IV
AD	21	M	80	15.5	Braak IV	/
	22	M	82	11.5	Braak IV	/
	23	M	85	31.08	A2B3C2	/
	24	F	83	25	A2B3C2	/
	25	M	91	25	A2B3C2	/
	26	F	96	26.5	Braak IV/V	/
	27	M	87	21.75	A2B3C2	/
	28	M	90	26.12	A3B2C3	/
	29	F	87	17	A1B2C2	/
	30	M	90	31.98	A2B2C3	/

AD = Alzheimer's Disease, CTL = age-matched controls, DLB = Dementia with Lewy Bodies, NFT = Neurofibrillary tangles, PMI = post-mortem interval, * = after neuropathological examination.

Table 2 Primary antibodies

Target	Epitope	Clone	Host species	Dilution	Manufacturer	Reference	RRID
P- α -Synuclein	P-Ser129	11A5	Mouse (M)	1:500	Prothema Biosciences*	NA	NA
P- α -Synuclein	P-Ser129	81A	Mouse (M)	1:200	Millipore	MABN826	AB_2904158
P- α -Synuclein	P-Ser129	EP1536Y	Rabbit (M)	1:200	Abcam	ab51253	AB_869973
Amyloid- β	AA17-24	4G8	Mouse (M)	1:200	BioLegend	800712	AB_2734548
Iba1/AIF-1	C-term	NA	Rabbit (P)	1:500	Wako	019-19741	AB_839504
NF-H	Met1-Ala380	NA	Goat (P)	1:500	Biotechne	AF3108	AB_2149640
P-Tau	Ser202, Thr205	AT8	Mouse (M)	1:500	ThermoFisher	MN1020	AB_223647
P-Tau	Ser422	NA	Rabbit (P)	1:150	ThermoFisher	44-764G	AB_1502115

M = monoclonal, NA = not available P = polyclonal.

* = Provided by Dr. Manuel Buttini.

Table 3 Secondary antibodies

Fluorophore	Host species	Reactivity	Isotype	Dilution	Manufacturer	Reference	RRID
Alexa Fluor 488	Donkey	Rabbit	IgG (H+L)	1:300	Jackson	711-545-152	AB_2313584
Alexa Fluor 488	Donkey	Mouse	IgG (H+L)	1:400	Jackson	715-545-150	AB_2340846
Alexa Fluor 488	Donkey	Goat	IgG (H+L)	1:400	Jackson	705-545-147	AB_2336933
Alexa Fluor 555	Donkey	Rabbit	IgG (H+L)	1:400	Invitrogen	#A-31572	AB_162543
Alexa Fluor 647	Donkey	Mouse	IgG (H+L)	1:300	Jackson	715-605-150	AB_2340862
Alexa Fluor 647	Donkey	Rabbit	IgG (H+L)	1:300	Jackson	711-605-152	AB_2492288

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6