Axonal degeneration in the anterior insular cortex in Parkinson’s disease and Dementia with Lewy bodies: more than just an α-synuclein story

Yasmine Y. Fathy (✉ y.fathy@vumc.nl)
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc
https://orcid.org/0000-0001-9236-2479

Laura E. Jonkman
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc

John J. Bol
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc

Evelien Timmermans
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc

Allert J. Jonker
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc

Annemieke J.M. Rozemuller
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc

Wilma DJ van de Berg
Vrije University Academic Hospital: Amsterdam UMC Locatie VUmc

Research Article

Keywords: α-synuclein, insular subregions, axonal length density, Alzheimer’s disease pathology, neurofilament, myelin

Posted Date: June 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1767866/v1

License: ☋ ☀ This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background

Axons, crucial for impulse transmission and cellular trafficking, are thought to be primary targets of neurodegeneration in Parkinson’s disease (PD) and Dementia with Lewy bodies (DLB). Axonal degeneration occurs early, preceeding and exceeding neuronal loss, and contributes to the spread of pathology, yet is poorly described outside the nigrostriatal ciruitry. The insula, a cortical brain hub, was recently discovered as highly vulnerable to pathology and plays a role in cognitive deficits in PD and DLB. The aim of this study was to evaluate morphological features and burden of proteinopathy and axonal degeneration in the anterior insular sub-regions in PD, PD with dementia (PDD), and DLB.

Methods

α-Synuclein (α-syn), phosphorylated (p-)tau, and amyloid-β pathology load were evaluated in the anterior insula (agranular and dysgranular) subregions of brain donors (n = 27). Axonal loss was evaluated using modified Bielschowsky silver staining and quantified using stereology. Cytoskeletal damage was comprehensively studied using immunofluorescent multi-labelling and 3D confocal laser-scanning microscopy.

Results

Compared to PD(D), DLB showed significantly higher α-syn and p-tau pathology load, argyrophilic grains, and most severe axonal loss, particularly in the anterior agranular insula. Alternatively, the dysgranular insula showed a significant higher load of amyloid-β pathology. Using mixed model analysis, p-tau contributed most to axonal loss in the DLB group, highest in the anterior agranular insula. Neurofilament and myelin showed degenerative changes including swellings, demyelination, and detachment of the axon-myelin unit.

Conclusions

Our results highlight the selective vulnerability of the anterior agranular insular sub-region to various converging pathologies, leading to impaired axonal integrity in PD(D) and DLB, disrupting its functional properties and potentially contributing to cognitive, emotional, and autonomic deficits.

Introduction

The presence of α-synuclein pathology and loss of dopaminergic neurons are known as the main neuropathological hallmarks of Parkinson’s disease (PD) and Dementia with Lewy bodies (DLB). PD and DLB are two of the most common and progressive neurodegenerative diseases affecting more than 6 million people worldwide, and both lack disease modifying treatments [1, 2]. Post-mortem human studies have shown that during the first 3 years of PD diagnosis, approximately 35–75% of striatal nerve terminals are already lost...
compared to the loss of dopaminergic neurons which lags behind [3, 4]. The magnitude of nerve terminal loss compared to that of neurons indicates that axons could be primary targets of degeneration while neurons die as a result of a "dying back" process [5, 6]. Axonal loss has generally been found to occur quite early [3, 4, 6–8], precedes neuronal loss [5, 9], and affects both the central and peripheral nervous systems [10, 11]. Pradoxically, axons are also thought to contribute to the spread of pathological aggregates through α-synuclein transport across synaptically connected regions [12]. Not all axons are equally vulnerable to degeneration; long, thin, densely arborized, and poorly myelinated axons of projection neurons were found most susceptible to Lewy pathology and degeneration [13–15]. These axons generally have high metabolic demands as well as proteostatic burden [16]. Overall, axonal damage and loss of connectivity are increasingly associated with cognitive dysfunction, thus posing as a potentially relevant biomarker for cognitive impairment in PD, a burdening multi-faceted aspect of the disease [17, 18].

According to our knowledge, the morphological features of axonal degeneration and myelin changes in PD and DLB have not yet been well-described outside the nigrostriatal circuitry. We recently discovered that the insular cortex, a brain hub with crucial integrative functions, is highly vulnerable to α-synuclein pathology in PD and DLB [19]. We observed a severe load of Lewy pathology and astroglial degenerative changes in the anterior insula most prominent in PD with dementia (PDD) and DLB donors. The insular cortex is broadly connected to the whole brain and plays a role in affective, autonomic, somatosensory, and cognitive functions [20, 21]. The multi-functional capabilities of the insular cortex are driven by an underlying heterogeneous cyto-architecture namely; Anterior (agranular and dysgranular) and posterior granular, each with its own set of preferential connections. The insular cortex is thought to widely contribute to non-motor deficits in PD and shows early changes in PD with mild cognitive impairment as well as prodromal PD and DLB [22–24].

In the current study, we comprehensively evaluated the morphological features of axonal and cytoskeletal degeneration, concomitant pathology, and their relationship with axonal loss in the anterior insular cortex. We hypothesized that axonal degeneration is most severe in the agranular compared to the dysgranular insula in PD(D) and DLB and associated with the local burden of α-synuclein, phosphorylated tau (p-tau), and amyloid-β pathology. In a well-characterized cohort of PD(D) and DLB brain donors (N = 27), we studied axonal degeneration using a modified Bielschowsky silver staining, multicolour immunofluorescence combined with advanced confocal laser-scanning microscopy (CSLM), and stereological principles. Using 3D microscopy, we provide an overview of morphological features of proteinopathy as well as axonal and cytoskeletal degeneration in the anterior insular subregions of PD(D) and DLB donors.

Materials And Methods

Post-mortem brain tissue

Formalin-fixed postmortem brain tissue from 27 donors with PD(D) and DLB (range = 60–93 years) was collected in close collaboration with the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands; www.brainbank.nl). In compliance with ethical and legal guidelines, all donors or next of kin had provided written informed consents for donation of brain tissue and access to their clinical and neuropathological reports for research purposes. The brain donor program of the NBB were approved by the local medical ethics committee of the VU University Medical Center, Amsterdam. The main inclusion criteria for the current study
were: (i) clinical diagnosis of PD, PD with dementia (PDD), or DLB according to revised MDS and McKeith diagnostic criteria [25, 26] and (ii) neuropathological confirmation of diagnosis [27]. Subjects were excluded if they had a long history of neuropsychiatric disorders or concomitant neurological disorders, and/or infarcts and other abnormalities within the insular cortex (Table 1).

Brain dissection was performed according to international guidelines of Brain Net Europe II (BNE) consortium (http://www.brainnet-europe.org) and NIA-AA by an experienced neuropathologist (NBB: AMR). The entire insula was dissected into 0.5–1 cm thick blocks according to its anatomical borders with surrounding brain regions. Using the central sulcus as a landmark separating the anterior from the posterior insula, the anterior insular cortex (AIC) was then dissected further [28]. Tissue blocks were cryo-protected with 30% sucrose, frozen and sectioned using a sliding microtome into 60μm-thick sections and these were stored at −30°C until further processing. Sections were first incubated in various gradients of alcohol followed by xylene to induce de-fatting then stained with Nissl. This allowed the identification of the agranular and dysgranular sub-regions based on the absence or presence of layers II and IV by two experienced researchers (YF and LJ) as previously described [19, 29].

Neuropathological assessment

For neuropathological diagnosis and staging, 6 μm paraffin sections from brainstem, limbic and neocortical brain tissue blocks of all donors were immunostained for α-synuclein (clone KM51, 1:500, Monosan Xtra, The Netherlands), amyloid-b (clone 6F/3D, 1:500, Dako, Denmark) , phosphorylated tau (p-tau, clone AT8, 1:500, Thermo Fisher Scientific, USA), haematoxylin and eosin (H&E), TDP-43 and congo red according to current diagnostic guidelines of BrainNet Europe [27]. Pathological staging protocols were based on Braak α-synuclein (Braak α-syn 0–6), Braak staging for neurofibrillary tangles (Braak NFT 0–6), Thal phase for amyloid-β (0–5), and CERAD score for neuritic plaques [30-34]. Glial tauopathy such as age-related tauopathy of the astroglia (ARTAG) and primary age-related tauopathy (PART) were assessed primarily in the temporal cortex, olfactory cortex and amygdala by an experienced neuropathologist (AMR) [35, 36]. Clinical symptoms and diagnosis ante-mortem, along with neuropathological scores, were used to provide a final pathological diagnosis (Table 1).

Evaluation of axonal degeneration using modified Bielschowsky silver staining

A modified Bielschowsky silver staining [37] was used for the evaluation of axonal degeneration in the anterior insula. Prior to staining, all glassware was thoroughly rinsed with purified water for several hours (h) or overnight and metals were avoided. All incubations were performed at cold temperature (5°C) [37]. Free-floating 60-μm tissue sections (interval: 1 in 20) were rinsed in distilled water followed by Tris buffered saline (TBS, pH 7.6) and incubated in 20% silver nitrate solution (AgNO₃; Sigma, CAS No: 7761-88-8) for 30 minutes at 5°C in the dark. Subsequently, non-evaporated ammonium hydroxide 30-33% (NH₃) solution was slowly added to the sections until the color turned black, then incubated for 30 minutes at 5°C in the dark (Honeywell, CAS No: 1336-21-6). Sections were then rinsed with evaporated ammonia-water for 10 minutes (100 µl ammonia in 50 ml distilled water). Subsequently, a developer solution was freshly prepared and consisted of 100 ml distilled water; 20 ml 37% formalin (Sigma, CAS No: 50-00-0), 1 drop nitric acid (Fisher, CAS No: 7697-37-2), 0.5 gr citric acid (Sigma, CAS No: 77-92-9). Sections were incubated in the developer solution at 5°C in the dark for
visualization of the reduced metallic silver. Staining was monitored carefully and the incubation was stopped within 10-15 minutes. This was followed by rinsing in Hypo (sodium thiosulfate 5% in distilled water; Honeywell, CAS No: 7772-98-7) to stabilize the developing reaction and remove free AgNO₃ deposits from the tissue. Sections were then rinsed, mounted with 0.3% gelatin (Oxoid), dried and cover-slipped using Entellan (Sigma).

**Evaluation of α-synuclein, p-tau, and amyloid-β pathology in the anterior insular subregions**

For α-synuclein immunostaining, consecutive free-floating 60-μm thick sections of the anterior insula were pre-treated with 98% formic acid (Sigma-Aldrich, Darmstadt, Germany) and incubated with primary antibody mouse anti-α-synuclein (syn-1, 1:2000, 610786, BD Biosciences, Berkshire, UK) in TBS and 0.5% TritonX-100 solution overnight at room temperature, as previously described by Braak and colleagues [38]. For immunostaining with antibodies against p-tau and amyloid-β, free-floating sections were rinsed with TBS and pre-treated with citrate buffer (pH 6.0) in a steamer (95°C) for antigen-retrieval, followed by 80% formic acid for amyloid-β. Non-specific staining was then blocked with 0.3% H2O2 and 0.1% sodium azide in TBS followed by 2% normal goat serum. Subsequently, sections were incubated in primary antibody mouse anti-p-tau (1:1000, MN1020, AT8, Thermofisher-scientific, Netherlands) and adjacent sections were incubated in mouse anti-amyloid-β (1:1000, M08720, clone 6f/3d, Dako, Denmark) diluted in TBS and 0.1% Triton-X overnight at 4°C. All sections were incubated with biotinylated secondary antibody IgG (1:200, Vector Laboratories, Burlingame, CA, USA) followed by standard avidin-biotin complex (1:200, Vectastatin ABC kit, Standard; Vector Laboratories) in TBS for 2h. Tissue samples were incubated in 3,3′-diaminobenzidine (DAB) to visualize staining and were mounted and counter-stained with thionin (0.13%, Sigma-Aldrich, Darmstadt, Germany).

Load of α-synuclein, p-tau and amyloid-b pathology was calculated as percentage immunoreactivity per surface area in each region of interest (ROI) using area fraction plugin in ImageJ (1.52n) [39]. Anterior insular sub-regions (agranular and dysgranular) were identified on adjacent Nissl-stained sections, and the same borders were used to define ROIs on sections immuno-stained for pathological aggregates [19].

**Multi-labelling immunofluorescence for evaluation of axonal cytoskeletal abnormalities**

We included multi-labelling immunofluorescent staining with axonal and myelin markers to visualize the axonal morphology and cytoskeletal abnormalities in 3D with CSLM. Adjacent 60-μm thick sections from PD(D) and DLB cases were rinsed in TBS, pre-treated with EDTA-Tris (pH 9.0) in a steamer (95°C) and incubated in a cocktail of the following primary antibodies: 1) mouse-anti myelin proteolipid protein (PLP, 1:500, MCA839G, plpc1, Bio-Rad, Netherlands) and 2) chicken anti-neurolament heavy chain (NfH, 1:500, AB5539, heavy-chain, Millipore) diluted in TBS, 2% normal donkey serum, and 0.5% Triton-X. Immunostaining was performed for 48h at 4°C followed by incubation with secondary antibody goat anti-chicken coupled with Alexa Fluor 594 (1:400; Molecular Probes, Waltham, MA, USA), donkey anti-mouse coupled with Alexa Fluor 647, and dianmidino-2-phenylindole(4,6)dihydrochloride (DAPI; Sigma) for nuclear staining for 2h in the dark. Tissue samples were subsequently rinsed in TBS and blocked in 5% normal mouse serum for 1hr followed by incubation with Alexa488-conjugated mouse anti-phosphorylated-Serine129 (pSer129) α-synuclein antibody
(1:100,11A5, gift from Prothena Biosciences Inc., USA) for 2h at 4°C in the dark. The tissue sections were then mounted on glass slides and cover-slipped with mowiol-DABCO as a mounting medium (4-88 Calbiochem).

**Microscopic imaging**

Digital images of the immunostained slides were made with a photomicroscope (Leica DM5000) equipped with color camera (DFC450), Leica LASV4.4 software and 63× oil objective lens. Immunofluorescent labelling was visualized using CLSM LEICA TCS SP8 (Leica Microsystems, Jena, Germany). Adjacent Nissl-stained sections were used for the delineation of subregions and the ROIs were superimposed on the images of the immunofluorescent stained sections. This was followed by sampling of all axons (Bielschowsky staining) in the superficial and deep layers of each sub-region. Image acquisition was done using 100×/1.4 NA objective lens, 405 nm diode, and pulsed white light laser (80 Hz) with excitation wavelengths 405, 499, 598, and 653 nm, scanned using frame/stack sequential mode. For optimal resolution, z-step size was calculated for each scan based on the Nyquist-Shannon sampling theorem [40] and line accumulation/averaging were used as deemed appropriate per channel. Deconvolution of image stacks was performed using Huygens Professional software (Scientific Volume Imaging, Hilversum, the Netherlands). Images are shown as maximum projections of all channels combined and all figures were composed using Adobe Photoshop (Adobe Systems Incorporated).

**Stereological analysis of axonal loss**

Axonal length density, based on Bielschowsky silver impregnated sections, was quantified using the stereological space balls probe from stereoinvestigator software (MBF Bioscience, Williston, VT, USA) and Leica microscope DMR HC (v2019.01.4) [41-43]. Serial sections (1:20, Range of sections: 3-8) were used for quantification, and counting parameters were chosen to allow counting ≥200 axon intersections for each sub-region. A sphere (radius=10μm) was used along with a sampling grid 2700 x 2700μm. ROIs corresponding to the grey matter of the agranular and dysgranular sub-regions were drawn at a low magnification (using a 2.5x objective) and axonal quantification was completed at higher magnification (100x oil objective). Only nerve fibers were counted and when a counting frame/sphere contained tangles or other pathologies, they were not counted and the following counting frame was used. Moreover, a fiber was counted only when it fully intersected with the sphere at least once. Tissue thickness was measured manually at each sampling frame (mean= 22 mm ± 1.42) and coefficient of error (CE) was calculated for each sub-region (mean CE agranular=0.13 ± 0.06; CE dysgranular = 0.12 ± 0.05).

To calculate estimated axonal length (L), total number of intersections of fibers with space balls (Qi) throughout all sections is multiplied by the volume of sampling frame. The volume (V= grid X * grid Y * section thickness) is divided by the surface area of sphere (a=) multiplied by the reciprocal of sampling fraction of the section (1:20) [41, 43]. To calculate density (mm/mm³), total axonal length (L) was divided by sampled reference volume per ROI. Reference volume was derived through planimetry; calculated as a measure of total area of the ROI multiplied by section height [41, 43].

**Statistical Analysis**
Statistical analyses were performed using SPSS (version 26.0, IBM) and graphs were made using graph prism, version 9. When tissue or staining quality was insufficient, cases were omitted from analysis. Demographics of controls, PD(D) and DLB were compared using chi-square test for categorical data and ANOVA, with age corrections, for numerical data. To analyze differences in axonal length density between insular sub-regions (agranular and dysgranular) across all three patient groups (PD, PDD, DLB), an ANCOVA, with adjustment for age, and post-hoc paired t-test were used. Quantitative scores for p-tau and a-synuclein pathology load showed non-normal distribution and were log transformed followed by parametric analysis using ANCOVA and age as covariate with post-hoc paired t-test for group differences. For amyloid-β, due to the presence of multiple zero scores from pathology quantification, scores were dichotomized (0 or 1) and analyzed using logistic regression. To assess the pathology and group effects on axonal density, variables were pooled into the model and a nested linear mixed model analysis was performed using backward elimination. Axonal length density was the dependent variable, area% load of p-tau, a-synuclein, and amyloid-β were main effects and age was a covariate. Correction for multiple comparison was not performed. Statistical significance was set at p<0.05.

Results

Cohort description

All included donors had moderate to severe disability (estimated H&Y=4-5). DLB donors were significantly younger than PDD donors (mean= 71±6.1 & 81.4±8.4, respectively; p=0.02) and had a significantly shorter disease duration (mean=5±2.4) compared to both PD and PDD (mean=18±4 and 13.4±5 years, respectively; p=0.001). All disease groups had advanced Braak α-synuclein stages, whereas Braak NFT stages were most advanced in PDD as well as DLB and accompanied by glial tauopathy (ARTAG). The DLB group also had advanced amyloid-β Thal phases (range: 3-5) except for one younger patient (DLB_5, Thal phase=0). Cerebral Amyloid angiopathy (CAA) was more frequent (66% of cases) in the DLB group compared to PDD (25%) and PD (20%) and included both type I and type II CAA. The demographics, age-of-onset, disease duration and neuropathological staging of all donors included in this study are summarized (Table 1).

Burden of α-synuclein, p-tau, and amyloid-β pathology

Comparing between the PD, PDD and DLB groups, we observed significant differences in the load of α-synuclein pathology in the dysgranular insula (F(2,18)=4.9, p=0.019) and a trend for the agranular insula (F(2,18)=3.5, p=0.054). The DLB group showed significantly higher scores compared to PD and PDD in both subregions (p=0.016 and 0.015 for dysgranular region; p=0.02 for agranular region; Figure 1). No difference was found between PD and PDD (p=0.86). Between the agranular and dysgranular insula, α-synuclein pathology load was significantly higher in the agranular insula in all groups (mean α-synuclein % area = 3.25% (agranular) and 1.55% (dysgranular); t(21)=5.3, p<0.001). For p-tau, we also observed a significant difference between disease groups for both the agranular (F(2,19)=5.5, p=0.01) and dysgranular insula (F(2,19)=7.1, p=0.005). The DLB group showed a significantly higher load of p-tau compared to PD and PDD groups for both sub-regions (agranular: p=0.01 (PD) and p=0.006 (PDD); dysgranular: p=0.004 (compared to both groups). Comparing p-tau pathology within insula sub-regions across groups, the agranular insula showed significantly higher p-tau load compared to the dysgranular insula (mean= 18.0% and 7.8%, respectively;
t(19)=5.1, \( p<0.001 \). There were no group differences for amyloid-\( \beta \) pathology \( (X^2(2)=3, p=0.22; X^2(2)=2.6; p=0.26) \). However, comparing anterior insula sub-regions across groups, the dysgranular insula (mean=5.0% ± 4.8%) showed significantly higher amyloid-\( \beta \) load compared to the agranular insula (mean=3.0% ± 8.3%; \( t(25)=-2.2, p=0.037 \) (Figure 1).

**Morphology of \( \alpha \)-synuclein, p-tau, and amyloid-\( \beta \) pathology**

We observed Lewy bodies (LBs), mostly in pyramidal neurons of deeper layers (V-VI), and LNs including bulgy LNs (Figure 2; a-c) across all layers in both subregions. Cytoplasmic granular \( \alpha \)-synuclein deposits (d-e), string-shaped and circular \( \alpha \)-synuclein deposits (f-g) as well as astrocytic synucleinopathy (h-j) were observed, particularly in the agranular insula of PD(D) donors. The anterior insula in DLB donors showed more severe \( \alpha \)-synuclein pathology with extensively dense LNs and astroglial star-shaped synucleinopathy (k-m). In one PDD donor, vascular synucleinopathy was observed in the anterior insula (h-j). Furthermore by staining astrocytes, \( \alpha \)-synucleinopathy was found within the GFAP-positive astrocyte cell bodies and surrounding processes in both sub-regions (n-p).

The anterior insula of the PD(D) and DLB donors showed various degrees of severity for p-tau and amyloid-\( \beta \) pathology. NFTs, ghost tangles, glial tauopathy (including ARTAG and subpial thorny astrocytes), and neuritic plaques were observed in both sub-regions (Fig. 3: a-l). We also observed fragmentation of NFTs in the agranular insula along with argyrophilic grain disease (AGD) deposits in DLB (e-l). p-Tau pathology was also observed in fork cells in deeper layers of the agranular insula (b). Neuropil threads, ghost tangles, and neuritic plaques were most prominent in DLB. Amyloid-\( \beta \) plaques were observed in the dysgranular insula and comprised of diffuse plaques as well as dense core plaques. Meningeal amyloid-\( \beta \), CAA type I and II, and dyshorric amyloid (i.e. perivascular amyloid deposits) were most prominent in the dysgranular insula in DLB patients (Figure 3; m-u).

In summary, across groups, the agranular insular showed more severe \( \alpha \)-synuclein and p-tau, but not amyloid-\( \beta \) pathology compared to the dysgranular sub-region of the anterior insula. Furthermore, \( \alpha \)-synuclein pathology in the dysgranular insula was significantly different between DLB and PDD; while p-tau was higher in DLB compared to both PD and PDD. For amyloid-\( \beta \), no differences between groups were observed.

**Axonal length density is significantly reduced in the agranular insula**

Taking all groups together, mean total axonal length density (mm/ mm\(^3\)) for the agranular and dysgranular insula were 8.00 ±2.14 m/m\(^3\) and 10.6 ±2.86 m/m\(^3\), respectively. Axonal length density was significantly higher in the dysgranular compared to the agranular insula among all groups (t(23)=-2.7; \( p=0.01 \) and \( t(23)=-5.7; p<0.001 \)). A significant effect of disease group on axonal length density was only observed in the dysgranular sub-region (F(2,23)=3.7; \( p=0.04 \); agranular: F(2,23)=2.5; \( p=0.11 \)). Specifically, a significant reduction was found in DLB (8.66 m/m\(^3\)) compared to PD (11.75 m/m\(^3\)) and PDD donors (11.94 m/m\(^3\)) (\( p=0.04 \) and 0.02, respectively) (Figure 1).

**Morphological features of axonal degeneration in the anterior insular sub-regions**
Neurodegenerative features such as localized axonal thickening, thinning, spheroids and myelin abnormalities were observed in the agranular insula in all donors (Figure 4). In cases with more advanced Thal phases, amyloid-β plaques surrounding axons and bulbous axonal swellings were observed in the vicinity of the plaque (Fig.4; h-j). In PD donors, early NFT formation, pre-tangles, were seen associated with dystrophic neurites (Fig.4; k-m). NFTs maturing to ghost tangles were particularly present in DLB donors, where severe axonal degeneration and AGD deposits, seen as comma shaped deposits, were commonly seen (Figure 4; o-s).

To assess cytoskeletal changes in the anterior insular subregions in PD(D) and DLB donors, we studied morphological changes in the pattern of NfH and PLP (myelin) immunoreactivity. We observed blister-like myelin formations around thickened axonal segments in both sub-regions, not associated with pSer129 α-synuclein aggregates in all disease groups (Fig.5; a,j). In DLB, severe myelin changes were observed including swellings (containing multiple vacuoles), demyelination, and myelin blisters with disrupted axon-myelin continuity (Fig.5; a,j,k). NfH features consisted of fragmentation, extracellular debris and spheroids with single axonal connections, indicating axonal transection (Fig.5; d-g). Extracellular encagement of LBs and donut-shaped NfH aggregates were also observed (Fig 5; c&h). Bulgy LNs were seen in myelinated fibers as well as non-myelinated fibers in the agranular and dysgranular insular sub-regions (Figure 5; b,l).

**Tau pathology is a major contributor to axonal loss in the anterior insula**

Using a linear mixed model including all pooled variables (axonal density, disease group, age, p-tau, amyloid-β, and α-synuclein pathology load), p-tau load showed the highest association with axonal length density in both anterior insular subregions (b=-3.45x10^-5; F(1,42)= 4.2; p=0.046). Post-hoc analysis showed a significant effect for p-tau on axonal length density in the DLB group (b=-5.04x10^-5; t(24.2)=-3.24, p<0.001) compared to PD (b=-1.5x10^-5; t(40.6)=-0.42, p=0.67) and PDD (b=-6.4x10^-5; t(41.3)=-0.7, p=0.5) as well as for the agranular compared to dysgranular insula (b=-5x10^-5; t(42.3)=-3, p=0.004). Testing other pathology variables effect on axonal density showed a significant effect for α-synuclein (b=-3x10^-4; t(29)=-3, p=0.006) but no significant effects for amyloid-β (p=0.77). Post-hoc analysis for group and sub-region on axonal length density showed significant effects for the DLB group (b=-3.34x10^-4; t(45.7)=-3.3, p=0.002) compared to PD (p=0.4) and PDD (p=0.7) and agranular compared to dysgranular sub-region (b=-3x10^-4; t(36.6)=-2.7, p=0.009) (Figure 1).

**Discussion**

The anterior insular cortex is an important brain region widely connected to the brain, with crucial integrative functions including affective, cognitive, homeostatic, and somatosensory [44]. These integrative functions, particularly of the anterior insula, are mainly the result of dense intra/inter axonal insular connections which are disrupted and known to be associated with clinical symptoms in PD [45]. Yet, little is known regarding the insula's underlying neuropathological and degenerative characteristics in PD(D) and DLB, information of which could aid in the identification of primary causes and targets of neurodegeneration. In this study, we aimed to assess axonal degeneration and its relationship with various pathological aggregates in the anterior insular cortex sub-regions in PD(D) and DLB. α-Synuclein and tau pathology load were most severe in the agranular insula of all patient groups, and highest in DLB, while amyloid-β pathology was more severe in the
dysgranular insula. In general, the anterior insula invariably harbored a constellation of pathologies including neuronal and astrocytic synucleinopathy, pre-tangles, mature ghost tangles, astrocytic tauopathy, diffuse and dense core amyloid-β plaques, CAA (type I and II) and AGD. p-Tau pathology was found to have the most significant effect on axonal loss, which was most pronounced in the DLB group. Features of axonal degeneration included fragmentation, thinning, thickening, and spheroids as well as cytoskeletal abnormalities including neurofilament degeneration, detachment of the axon-myelin unit and demyelination.

The anterior agranular insula has a distinct allocortical architecture with only four layers, less myelinated fibers as well as late myelination during brain development [46]. Several features may predispose the agranular anterior insula to early degeneration, such as severe impairment of the protein degradation systems and inflammation in response to protein toxicity, leading to build-up of protein aggregates [47]. Moreover, poorly myelinated or unmyelinated fibers are considered more vulnerable to pathology and degeneration, not only in PD but also in other neurodegenerative diseases. The late myelination of the anterior insula during brain development predisposes it to degeneration, as early myelination provides protection to axons through salutatory conduction reducing the metabolic demands of the neuron and thus oxidative stress [11, 48]. In this study, the agranular anterior insula exhibited more vulnerability to α-synuclein and p-tau pathology, the latter of which had more significant effect on axonal loss, than the dysgranular area. α-Synuclein and p-tau pathology has been shown to co-occur in the same neurons, the two proteins can interact together and α-synuclein aggregation is thought to induce the phosphorylation of tau, thus promoting tauopathy [49]. This would result in detachment of tau from the cytoskeleton, depolymerization of the microtubule system, tau aggregation and neurofibrillary tangle formation leading to impaired axonal transport, synaptic loss, and neuronal loss [50].

Amyloid-β pathology, on the other hand, was more prominent in the anterior dysgranular insula, compared to the agranular sub-region. Amyloid-β forms extracellular plaques which mechanically disrupt the function of axons and neurons [51]. Dystrophic neurites found near or within plaques have shown axonal transport deficits contributing to synaptic deficits [52, 53]. Likewise, the early damage of axons and deficient axonal transport would lead to organelle and phagosome accumulation, thereby disrupting protein clearance mechanisms [54, 55]. Accumulating pathology also has an influence on the stability of myelin leading to further loss of axonal maintenance and reduced neurofilament transport [56]. Of note, recent evidence shows that approximately 40% of amyloid-β is produced in axons [57, 58]. Put together, this is particularly relevant in DLB where the co-occurrence of all three pathologies as well as CAA has commonly been reported [59]. The presence of these concomitant pathologies is associated with more severe neurodegeneration and rapid disease course leading towards cognitive decline and dementia, to which pathology in the anterior insula would largely contribute [59–61]. Besides AD pathology, AGD, a form of tauopathy, was also found in the anterior insula in DLB. AGD has a typical anterior to posterior distribution gradient and is linked to cognitive deficits [62].

In this study, axonal loss, studied as axonal length density, was found most pronounced in the agranular insula in the DLB group compared to other patient groups. While axonal loss in the dysgranular insula was significantly higher in DLB compared to both PD and PDD. These results support recent MRI studies highlighting the importance of anterior insular degeneration in DLB, indicating that the anterior dorsal (dysgranular) insula shows early atrophy in DLB, and could function as a potential biomarker [63]. Using
bielschowsky silver impregnation, we observed various degenerative axonal features, including thickening, thinning, spheroids, and bulbous swellings in the vicinity of β-amyloid plaques, many of which were also observed with neurofilament-heavy chain (NfH) immunostaining (as summarized in Fig. 6). Moreover, we found NfH spheroids fully encapsulating LBs and axonal spheroids with single axonal connections, indicating axonal transection. Neurofilaments are important cytoskeletal components regulating axonal caliber and growth as well as allowing docking and transport of organelles. Hyperphosphorylation of neurofilament-light chain (NfL) is thought to cause aggregation, impaired docking of motor proteins as well as impaired transport [64]. Recently, it has been found that NfL is present within LBs forming a shell-like structure along with other cytoskeletal structures encapsulating accumulated materials within LBs, such as lipids and proteins [65, 66]. Meanwhile, serum NFL biomarker levels are thought to reflect axonal neurodegeneration and correlates with cognitive decline in PD and AD [67–69]. Here, we show that NfH is also severely affected in the anterior insula. NfH subunits generally increase within axons as they mature, they play an important role in axonal support by allowing axonal stability which is important in providing docking sites for interactions with motor proteins and thus providing neurons, particularly those with long axons, the opportunity to conserve energy. It is also known that abnormal NfH phosphorylation generally leads to slower axonal transport thus affecting disease pathogenesis [70].

Likewise, the myelin sheath is an important axonal component providing support through glial axonal maintenance, allowing transportation of neurofilament and phosphorylation leading to higher axonal caliber, and faster transmission through the nodes of ranvier [56]. In this study, we reported myelin blisters, detachment from the axon-myelin unit, with thickening and bleb-like formation of the underlying neurofilament, as well as presence of LNs in myelinated nerve fibers showing patches of demyelination (as summarized in Fig. 6). Myelin blisters have been documented in multiple sclerosis whereby they cause disruption of the axon-myelin unit triggering abnormal calcium signalling and myelin degeneration [71]. Generally, myelination within the insula shows a decreasing gradient from posterior to anterior with late and scarce myelination of the agranular anterior insula [72]. To our knowledge, myelin changes have not been well-described in PD and DLB, yet appear to undergo early degeneration thus warranting further analysis. We previously showed swollen axons and myelin deficits with cryogenic X-ray nanotomography and illustrated ultrastructural myelin deficits in the substantia nigra in PD [73]. In the current study, we show that similar axonal deficits are present in the anterior insula in PD(D) and DLB.

This study, although the first to elucidate features of axonal degeneration and pathology in the anterior insula in PD(D) and DLB, has several limitations. First, we have a limited sample size, particularly for the PD group. Most PD cases at time of autopsy have records of severe cognitive decline and/or dementia (PDD). As such, brain tissue of PD donors without dementia is scarce. Yet future studies with larger cohorts at different disease stages could shed more light on the progression of axonal degeneration across PD and DLB. Second, our study lacks controls. Although thirteen controls were carefully selected and processed, the majority did not fulfill the quality requirements for the Bielschowsky staining and were therefore excluded. This is likely the result of postmortem changes and tissue handling processes non-compliant with Bielschowsky impregnation specifications.

Conclusions
Our study provides evidence that in PD and DLB, the anterior insula is highly selectively vulnerable to pathological aggregates whereby it harbors a constellation of deposits including α-synuclein aggregates, NFT, amyloid-β plaques, CAA and AGD. Degenerative features included axonal swelling and transection along with cytoskeletal neurofilament and myelin abnormalities in the anterior insula of PD, PDD as well as DLB patients. While the agranular insula was severely affected with p-tau and α-synuclein aggregates, amyloid-β pathology showed preferential distribution in the dysgranular insula. These abnormalities were most severe in the DLB patient group, providing supportive neuropathological evidence of anterior insular damage in this disease. Owing to the importance of the anterior insula as a hub connecting with various brain regions and integrating emotional, autonomic, and cognitive functions, the insula provides a unique opportunity to understand cytoarchitecture-specific vulnerability to pathology, degeneration, and contribution to widespread functional deficits.

**Abbreviations**

AGD: argyrophillic grain disease  
ARTAG: age-related tauopathy of the astroglia  
CAA: cerebral amyloid angiopathy  
CE: coefficient of error  
CLSM: confocal laser-scanning microscopy  
DLB: dementia with Lewy bodies  
DAB: diaminobenzidine  
H&Y: Hoehn and Yahr  
MDS: Movement disorder society  
NBB: Netherlands brain bank  
NfH: neurofilament heavy-chain  
NfL: neurofilament light-chain  
NFT: neurofibrillary tangles  
PART: primary age-related tauopathy  
PD: Parkinson's disease  
PDD: Parkinson's disease dementia  
PLP: proteolipid protein
Declarations

Ethics approval and consent to participate

Postmortem human brain tissue from clinically diagnosed and neuropathologically verified donors with advanced PD was collected by the Netherlands Brain Bank (brainbank.nl). In compliance with all local ethical and legal guidelines, informed consent for brain autopsy and the use of brain tissue and clinical information for scientific research was given by either the donor or the next of kin. A reference to the published Code of Conduct for brain banking of BrainNet Europe (where NBB participated in) is provided in the text. The procedures of the NBB (Amsterdam, The Netherlands) were approved by the Institutional Review Board and Medical Ethical Board (METC) from the VU University Medical Center (VUmc), Amsterdam.

Consent for publication

Not applicable

Availability of data and material

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

Dr. Wilma van de Berg was financially supported in period 2019-2021 by grants from Amsterdam Neuroscience, Dutch Research council (ZonMW 70-73305-98-106; 70-73305-98-102; 40-46000-98-101), Stichting Parkinson Fonds (Insula 2014), MJ Fox foundation (17253) and Parkinson Association (2020-G01). Dr. Wilma van de Berg performed contract research and consultancy for Hoffmann-La Roche, Roche Tissue Diagnostics, Crossbeta Sciences and received research consumables from Hoffmann-La Roche and Prothena. All authors declare no conflict of interest.

Funding

This work was funded by a grant from the Stichting ParkinsonFonds, SPF 38000, project number: 105475.

Authors’ contributions

Conceptualization: YYF and WvdB; Methodology: YYF, JJB, ET, WvdB and AJ; Formal analysis and investigation: YYF, WvdB and AJMR; Writing-original draft preparation: YYF and WvdB; Writing-review and editing: all co-authors; Funding acquisition: FJJ and WvdB; Resources: WvdB; Supervision: WvdB.

Acknowledgements
We are grateful to all individuals that donated their brains to the Netherlands Brain Bank (NBB; www.brainbank.nl), without their precious donations, this work would not have been possible. We thank the team of the NBB, in particular Michiel Kooreman, for their cooperation and their help in the collection of brain tissue. The authors would like to thank John Breve for his help testing western blot, Joost Heuvelink for technical assistance, and Jos Twisk for statistical advice. We also thank Marko Popovic and the Advanced Optical Microscopy Core O|2 (www.ao2m.amsterdam) for support with confocal imaging. This research was supported by Stichting Parkinson Fonds, Netherlands.

References


Tables

Table 1. Demographics of and neuropathological diagnosis of donors
<table>
<thead>
<tr>
<th>Subject ID &amp; clinical diagnosis</th>
<th>Sex</th>
<th>Age at death (years)</th>
<th>Disease duration</th>
<th>Braak α-synuclein</th>
<th>Braak NFT &amp; age-related tauopathy</th>
<th>Thal phase</th>
<th>CAA</th>
<th>PMD (h:min)</th>
<th>ABC score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD_1</td>
<td>M</td>
<td>77</td>
<td>11</td>
<td>II</td>
<td>1</td>
<td>5:35</td>
<td></td>
<td></td>
<td>A1B1C0</td>
</tr>
<tr>
<td>PD_2</td>
<td>F</td>
<td>68</td>
<td>16</td>
<td>II</td>
<td>1</td>
<td>7:55</td>
<td></td>
<td></td>
<td>A1B1C0</td>
</tr>
<tr>
<td>PD_3</td>
<td>F</td>
<td>73</td>
<td>3</td>
<td>II, ARTAG</td>
<td>2</td>
<td>6:10</td>
<td></td>
<td></td>
<td>A1B1C0</td>
</tr>
<tr>
<td>PD_4</td>
<td>M</td>
<td>91</td>
<td>26</td>
<td>III, PART</td>
<td>3</td>
<td>4:00</td>
<td></td>
<td></td>
<td>A3B2C1</td>
</tr>
<tr>
<td>PD_5</td>
<td>M</td>
<td>57</td>
<td>16</td>
<td>0</td>
<td>1</td>
<td>06:35</td>
<td></td>
<td></td>
<td>A1B0C0</td>
</tr>
<tr>
<td>PDD-1</td>
<td>F</td>
<td>88</td>
<td>15</td>
<td>II, ARTAG and PART</td>
<td>0</td>
<td>05:35</td>
<td></td>
<td></td>
<td>A0B1C0</td>
</tr>
<tr>
<td>PDD-2</td>
<td>M</td>
<td>74</td>
<td>7</td>
<td>II, ARTAG</td>
<td>3</td>
<td>04:35</td>
<td></td>
<td></td>
<td>A2B1C0</td>
</tr>
<tr>
<td>PDD-3</td>
<td>F</td>
<td>74</td>
<td>13</td>
<td>III</td>
<td>3</td>
<td>03:45</td>
<td></td>
<td></td>
<td>A2B2C1</td>
</tr>
<tr>
<td>PDD-4</td>
<td>F</td>
<td>81</td>
<td>8</td>
<td>II, ARTAG</td>
<td>3</td>
<td>06:40</td>
<td></td>
<td></td>
<td>A2B1C1</td>
</tr>
<tr>
<td>PDD-5</td>
<td>M</td>
<td>75</td>
<td>6</td>
<td>II, ARTAG</td>
<td>1</td>
<td>03:35</td>
<td></td>
<td></td>
<td>A1B1C0</td>
</tr>
<tr>
<td>PDD-6</td>
<td>M</td>
<td>70</td>
<td>19</td>
<td>III, ARTAG</td>
<td>3</td>
<td>04:30</td>
<td></td>
<td></td>
<td>A2B2C0</td>
</tr>
<tr>
<td>PDD-7</td>
<td>F</td>
<td>88</td>
<td>6</td>
<td>II, ARTAG</td>
<td>1</td>
<td>06:05</td>
<td></td>
<td></td>
<td>A1B1C0</td>
</tr>
<tr>
<td>PDD_8</td>
<td>M</td>
<td>81</td>
<td>18</td>
<td>III, ARTAG &amp; PART</td>
<td>0</td>
<td>06:10</td>
<td></td>
<td></td>
<td>A0B2C0</td>
</tr>
<tr>
<td>PDD_9</td>
<td>F</td>
<td>83</td>
<td>14</td>
<td>IV, ARTAG &amp; PART</td>
<td>0</td>
<td>06:15</td>
<td></td>
<td></td>
<td>A0B2C0</td>
</tr>
<tr>
<td>PDD_10</td>
<td>M</td>
<td>71</td>
<td>26</td>
<td>II, ARTAG</td>
<td>1</td>
<td>13:50</td>
<td></td>
<td></td>
<td>A1B1C0</td>
</tr>
<tr>
<td>PDD_11</td>
<td>F</td>
<td>87</td>
<td>10</td>
<td>III, ARTAG</td>
<td>3</td>
<td>07:55</td>
<td></td>
<td></td>
<td>A2B2C0</td>
</tr>
<tr>
<td>PDD_12</td>
<td>M</td>
<td>94</td>
<td>18</td>
<td>III, ARTAG</td>
<td>2</td>
<td>06:30</td>
<td></td>
<td></td>
<td>A1B2C1</td>
</tr>
<tr>
<td>PDD_13</td>
<td>M</td>
<td>75</td>
<td>12</td>
<td>II, ARTAG</td>
<td>0</td>
<td>12:25</td>
<td></td>
<td></td>
<td>A0B1C0</td>
</tr>
<tr>
<td>DLB-1</td>
<td>M</td>
<td>67</td>
<td>3</td>
<td>V, ARTAG</td>
<td>5</td>
<td>05:20</td>
<td></td>
<td></td>
<td>A3B3C3</td>
</tr>
<tr>
<td>DLB-2</td>
<td>M</td>
<td>75</td>
<td>2</td>
<td>III, ARTAG</td>
<td>4</td>
<td>05:00</td>
<td></td>
<td></td>
<td>A2B2C3</td>
</tr>
<tr>
<td>DLB-3</td>
<td>M</td>
<td>81</td>
<td>4</td>
<td>III</td>
<td>3</td>
<td>08:21</td>
<td></td>
<td></td>
<td>A2B2C0</td>
</tr>
<tr>
<td>DLB_4</td>
<td>M</td>
<td>78</td>
<td>6</td>
<td>I, ARTAG</td>
<td>3</td>
<td>05:10</td>
<td></td>
<td></td>
<td>A2B1C0</td>
</tr>
<tr>
<td>Subject</td>
<td>Gender</td>
<td>Age</td>
<td>Months</td>
<td>Years</td>
<td>ABC</td>
<td>Thal phase</td>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>-----</td>
<td>--------</td>
<td>-------</td>
<td>-----</td>
<td>------------</td>
<td>-------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLB_5</td>
<td>M</td>
<td>60</td>
<td>7</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>08:30</td>
<td></td>
</tr>
<tr>
<td>DLB_6</td>
<td>M</td>
<td>70</td>
<td>5</td>
<td>6</td>
<td>III</td>
<td>4</td>
<td>type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>grade 1</td>
<td>grade 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLB_7</td>
<td>M</td>
<td>71</td>
<td>9</td>
<td>6</td>
<td>II</td>
<td>3</td>
<td>type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>grade 1</td>
<td>grade 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLB_8</td>
<td>F</td>
<td>77</td>
<td>4</td>
<td>6</td>
<td>V</td>
<td>4</td>
<td>type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stage 1</td>
<td>stage 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLB_9</td>
<td>F</td>
<td>67</td>
<td>3</td>
<td>5</td>
<td>III</td>
<td>4</td>
<td>type</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stage 1</td>
<td>stage 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. **Subject demographics and neuropathological staging.** ABC score: A-C(0-3; A: Amyloid plaque score, B: Braak NFT score, C: CERAD neuritic plaque score), ARTAG: Aging-related tau astrogliopathy, Braak NFT: 0-VI, Braak α-synuclein: 0-6, CAA: Cerebral amyloid angiopathy (types: I (capillary) or II (capillary and arteriolar), stages 1-3), DLB: Dementia with Lewy bodies, DD: disease duration, NFT: neurofibrillary tangles, PART: primary age-related tauopathy, PD: parkinson's disease, PDD: PD dementia, PMD: post-mortem delay (hours: minutes), Thal phase: 0-5

**Figures**
**Figure 1**

**α-Synuclein, p-tau and amyloid-β pathology load in the anterior insular subregions.** α-synuclein pathology load was significantly higher in DLB agranular insula (5.7%) compared to PD (1.2%) and PDD (2.3%); the agranular insula had significantly higher pathology load (3.25%) compared to the dysgranular insula (1.55%) (a). p-tau was significantly higher in DLB for both the agranular (41.3%) and dysgranular insula (23.7%) compared to PD (15.1% and 0.25%) and PDD (4% and 0.44%); agranular insula had significantly higher pathology load (18%) compared to the dysgranular insula (7.8%). Amyloid-β was not significantly different between groups but the dysgranular insula had significantly higher pathology load (5%) compared to the agranular insula (3%)
(c). Axonal length density based on Bielschowsky silver staining and stereological count showed significantly lower density in the agranular (8 m/m³) compared to the dysgranular insula (10 m/m³). The DLB group had significantly lower axonal length density in the agranular insula (6.73 m/m³) compared to the PDD group (9.17 m/m³) and in the dysgranular insula compared to both PD and PDD (11.75 m/m³ and 11.94 m/m³ respectively); (d). Significance: * p<0.05, **p<0.01, *** p<0.001. AD: Alzheimer's disease; DLB: dementia with Lewy bodies; p-tau: hyperphosphorylated tau; PD: Parkinson's disease; PDD: Parkinson's disease dementia
Morphological features of α-synuclein pathology (syn-1) in the anterior insula of PD(D) and DLB patients. In the agranular insula, LNs in a PD_4 (a), a bulgy LN in PDD_1 (b), and in layer I in DLB_2 (c). Various LBs were observed in layer 5-6 and intracytoplasmic granular deposits in PD_5 and PD_3 (d,e), an α-synuclein double-string related to a cell, possibly glial, and surrounding a neuronal soma in PDD_1 (f), and circular deposit in a DLB_3 (g). Glial synucleinopathy (*) and a long LN were seen in PDD_1 in deeper layers of agranular insula (h). Vascular synucleinopathy (>) and glial synucleinopathy were seen in PDD-3 (i,j). In DLB subjects, dense α-synuclein deposits were seen in agranular insula (DLB_8, k), LNs and LBs in the agranular insular in DLB_1 and in the dysgranular insula (top image, DLB_2) (l), and star-shaped astrocytic synucleinopathy (*) surrounded by iron deposits in dysgranular insula in DLB_2 (<, m). Immunofluorescent staining of astroglial synucleinopathy (GFAP in red; α-synuclein in green) in the agranular insula in PD (n), in PDD_1 (o), and in DLB_2 (p) illustrates the extent of glial pathology in PD and DLB. LBs: Magnification (630x), scale bar: 50μm. DLB: dementia with Lewy bodies; LBs: Lewy bodies; LNs: Lewy neurites; PD: Parkinson’s disease; PDD: Parkinson’s disease dementia
Figure 3

Alzheimer’s associated pathologies in the anterior insula of PD(D) and DLB donors. In PD_3, a neurofibrillary tangle (NFT) is seen with distal swellings (a*, c), in PD-4, HPF-tau immunoreactive (AT-8 Ab, innogenetics) fork cell (b) and subpial thorny astrocytes in the agranular insula were seen also in PD_3 (d). NFTs with fragmentation of distal fibers were observed in PDD_1 and PDD_4 (e-h). In DLB_2, more severe neuropil threads, NFT with fragmentation (i) ghost tangles (j), neuritic plaque in DLB_1 (k), and glial tauopathy in DLB_2 (l) were seen. For amyloid-b (M08720, Dako), subpial pathology (m) and diffuse plaques (n) were seen in dysgranular insula in PD-4. In DLB_8, diffuse plaques with partial densification (o) and classic plaques (p)
were seen. In PDD-3, CAA in dysgranular insula (q) and blood vessels surrounded by perivascular amyloid-b plaques (r) in agranular insula were seen. In DLB-8, peri-vascular and vascular amyloid-β pathology (s-u) were found surrounded by amyloid-β plaques. Magnification 630x; Scale bar: 50μm. CAA: Cerebral amyloid angiopathy; DLB: dementia with Lewy bodies; NFT: neurofibrillary tangles; PD: Parkinson's disease; PDD: Parkinson's disease dementia.

Figure 4
Morphological features of axonal degeneration and associated pathologies in the anterior isula visualized using Bielschowsky silver staining. Axonal thinning (arrow) and associated myelin changes were visible in fibers in PD_5 (a-c). Axonal thickening is seen in PD_4 dysgranular insula (d), thinning and spheroid body in PDD_1 agranular insula (e), and DLB_5 and DLB_7 in both sub-regions (f,g). Multiple fibers were surrounded by β-amyloid plaques in PD_3 (j) and PDD_3 with bulbous swelling in nearby axons in agranular insula (h,i). Early NFTs are seen in PD_3 dysgranular insula (k) and in PDD_6 agranular insula where dystrophic neurites and peri-somatic granules are also present (l,m). In DLB_9, multiple perivascular amyloid-b plaques are visible (n). NFTs as well as severe axonal degeneration are present in DLB_1 (o) and in DLB_8 (r) and DLB_9 showing ghost tangles (p,q) surrounded by AG (p,r,s; upper right corner). AG are featured as comma shaped deposits. Magnification 630x; Scale bar: 50μm. AG: argyrophilic grains; DLB: dementia with Lewy bodies; NFTs: neurofibrillary tangles; PD: Parkinson's disease; PDD: Parkinson's disease dementia
Cytoskeletal (neurofilament; anti-NfH; red) and myelin (anti-PLP; yellow) degenerative changes in PD(D) and DLB donors. In PD-3, myelin blister and detachment from axon (a), LN showing α-synuclein deposits (green) in myelinated fiber, showing areas of demyelination in the dysgranular insula (b), and in the agranular insula a LB (green) surrounded by NfH donut are seen (c). NfH fragmentation (d,e) surrounded by myelin and α-synuclein debris (green) as well as debris and swelling (f) were visible in the dysgranular insula in PDD-4. In DLB_2 agranular insula, a large NfH swelling was visible surrounded by α-synuclein deposits (g), NfH donut (h), NfH fragmentation, swelling (*) and debris surrounded by α-synuclein aggregates (> , i), and focal axonal
thickening with myelin blister (j) were seen. In DLB_1 myelin swelling was seen with cross-section through the swelling showing multiple vacuoles (k). A long LN showing α-synuclein was also present in DLB_2. Magnification 1000x, scale bar: 10μm. DLB: dementia with Lewy bodies; LB: Lewy body; LN: Lewy neurite; NfH: neurofilament; PD: Parkinson’s disease; PDD: Parkinson’s disease with dementia; PLP: proteolipid protein in myelin.

Figure 6

Schematic drawing of proteinopathy and axonal degeneration in the anterior insula in PD(D) and DLB. The neuron, its axon, axonal cytoskeleton, myelin, and supportive cells such as astrocytes are all involved in the degenerative process in PD(D) and DLB with more severe features in DLB. Astrocytes are reactive and take up α-synuclein deposits ending in their degeneration. The axon is affected at multiple levels, it contains α-synuclein deposits (LBs and LNs) which impede axonal transport. Neurofibrillary tangles (NFT) contain hyperphosphorylated tau (p-tau) that begin as pre-tangles and mature into ghost tangles. The hyperphosphorylation and accumulation of p-tau impairs the axonal cytoskeleton and leads to depolymerization of the microtubule. p-Tau and α-synuclein can also stimulate each other’s aggregation speeding up degeneration. Amyloid-β plaques which are more severe in the dysgranular insula contributes to impaired cellular trafficking and myelin instability. Amyloid-β deposits in and surrounding blood vessels, CAA, lead to poor clearance of abnormal proteins and blood brain barrier dysfunction. Finally, myelinated axons are also affected through axon-myelin unit detachment and demyelination which affects conduction velocity and axonal support. All together, axonal length, integrity and cellular trafficking become impaired in the anterior insular subregions in PD, PDD and most severe in DLB donors with consequent impairment of insular functions as a brain hub and emotional/cognitive deficits as a result CAA: cerebrovascular amyloid angiopathy; DLB: Dementia with Lewy bodies; LB: Lewy body; LN: Lewy neurite; PD: Parkinson's disease; p-tau: hyperphosphorylated tau.