Evidence from spatial pattern analysis for the anatomical spread of \(\alpha\)-synuclein pathology in Parkinson’s disease dementia

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DOI: https://doi.org/10.5114/fn.2017.66710

Abstract
The objective of this study was to determine whether there is evidence from quantitative morphometry and spatial pattern analysis to support the hypothesis of anatomical spread of \(\alpha\)-synuclein in Parkinson’s disease dementia (PDD). Hence, clustering of \(\alpha\)-synuclein-immunoreactive Lewy bodies (LB), Lewy neurites (LN), and Lewy grains (LG) was studied in \(\alpha\)-synuclein-immunolabeled sections of cortical and limbic regions in 12 cases of PDD. The data suggested that: (1) LB, LN, and LG occurred in clusters which in 63% of regions were regularly distributed parallel to the tissue boundary, (2) in approximately 30% of cortical regions, the estimated cluster size of LB, LN, and LG was within the size range of cellular columns associated with the cortico-cortical pathways, (3) regularly distributed clusters were present in anatomically connected regions, and (4) the clustering pattern was similar to that of prion protein (PrP\(^{sc}\)) deposits in Creutzfeldt-Jacob disease (CJD). The clustering patterns of LB, LN, and LG were similar to those exhibited by cellular inclusions in other synucleinopathies and by PrP\(^{sc}\) deposits in prion disease and therefore, anatomical spread of pathogenic \(\alpha\)-synuclein could be involved in the pathogenesis of PDD.

Key words: Parkinson disease dementia (PDD), synucleinopathy, Lewy body (LB), Lewy neurite (LN), Lewy grain (LG), spatial pattern, anatomical spread.

Introduction
The ‘synucleinopathies’, viz. Parkinson’s disease dementia (PDD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) are characterized by the formation of cellular inclusions containing pathological forms of the pre-synaptic protein \(\alpha\)-synuclein. These pathologies include Lewy bodies (LB), Lewy neurites (LN), and Lewy grains (LG) in PDD and DLB [33] and glial cytoplasmic inclusions (GCI) in MSA [31]. \(\alpha\)-Synuclein is a small molecular weight protein which regulates the functioning of dopamine transporter and tyrosine hydroxylase [27]. In normal brain, monomers of \(\alpha\)-synuclein are unfolded soluble proteins, the oligomeric and fibrillar species existing in equilibrium. In the synucleinopathies, however, aggregation of \(\alpha\)-synuclein occurs to form \(\beta\)-sheet configurations rich in amyloid [34] resulting in the formation of pathogenic inclusions such as LB.

Pathogenic \(\alpha\)-synuclein may be secreted from cells, enter other cells, and seed small intracellular aggregates to form larger inclusions [23,35,37]. Hence, in PDD, pathological \(\alpha\)-synuclein may exhibit ‘prion-like’ behaviour and propagate through the brain via anatomical connections. In the prion dis-
ease, Creutzfeldt-Jakob disease (CJD), in which propagation of prion protein (PrPSC) along anatomical pathways is well established [12,13], PrPSC deposits are clustered in the cerebral cortex, the clusters often being regularly distributed parallel to the pia mater [7,9]. Clustering could therefore be a consequence of PrPSC spreading among regions via anatomical pathways [7]. To determine whether similar clustering of α-synuclein pathology occurs in PDD, the spatial patterns of LB, LN, and LG were studied in cortical and limbic regions in twelve cases of PDD.

**Material and methods**

**Cases**

Parkinson’s disease dementia cases (n = 12, details in Table I) were consecutive cases meeting the diagnostic criteria for Parkinson’s disease (PD) and PDD and were obtained from the Movement Disorders Center at Washington University School of Medicine in St. Louis [11,26]. Initial diagnosis of PD was based on modified United Kingdom Parkinson Disease Society Brain Bank (UKPDSBB) clinical diagnostic criteria [24]: (1) bradykinesia was present in association with at least one of the following, viz. rigidity, 4-6 Hz rest tremor, or postural instability, (2) three supporting criteria were also present such as unilateral onset, rest tremor, a progressive disorder, persistent asymmetry, a clinical condition present for more than 10 years, or excellent response to levodopa, and (3) the various exclusion criteria were applied such as the absence of stroke, persistent head injury, definite encephalitis, ocular crisis, family history of the disease, sustained remission, unilateral features after three years, progressive supranuclear palsy (PSP), early severe autonomic involvement, early signs of dementia, Babinski sign, cerebral tumor, exposure to MPTP, and negative response to levodopa [24]. Age of PD onset was determined by chart review and defined by onset of motor symptoms. Dementia in PD was determined by clinical assessment including the presence of impairment of attention, memory, executive, and visuo-spatial function together with behavioural disturbance, hallucinations and apathy [20]. The main pathological correlate of PDD is the presence of Lewy-body type degeneration in the cerebral cortex and limbic regions [20]. In addition, PDD cases are frequently associated with Alzheimer’s disease neuropathologic change (ADNC) and the degree of ADNC was assessed using National Institute on Aging-Alzheimer’s (NIA-AA) association guidelines ‘ABC’ [25]. This system uses four-point scales to assess the abundance of β-amyloid (Aβ) deposits.

**Table I.** Demographic features, duration of disease, and disease stage of the twelve cases of Parkinson disease dementia (PDD). Lewy body (LB) stage was assessed using a PD staging scale (range: 0, 1-6) and the McKeith et al. staging scale (McKeith et al. 1996, 2005). Alzheimer’s disease neuropathologic change (ADNC) was rated using the National Institute on Aging-Alzheimer’s (NIA-AA) association guidelines: A stage indicating β-amyloid (Aβ) deposition, B stage frequency of tau-immunoreactive neurofibrillary tangles (NFT), and C stage frequency of neuritic plaques (NP)

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('A' score), neurofibrillary tangles (NFT) ('B' score), and neuritic plaques (NP) ('C' score), the distribution of the three scores equating to four levels of ADNC, viz. 'not', 'low', 'intermediate', and 'high'. The 12 PDD cases in the present study equated to levels of ADNC from 'not' to 'intermediate' levels of AD.

**Tissue preparation**

After death, the consent of next-of-kin was obtained for brain removal, following local Institutional Review Board procedures and the 1995 Declaration of Helsinki (as modified in Edinburgh, 2000). Brains were fixed in 10% neutral buffered formalin for two weeks, paraffin-embedded, and sections cut at 6 mm. Blocks were taken from frontal and temporal lobes, the latter including the amygdala, hippocampus (HC), and dentate gyrus (DG). Histologic stains included hematoxylin and eosin and modified Bielschowsky silver impregnation. Immunohistochemistry was performed using the following antibodies: Aβ (10D5, 1:100,000; Elan Pharmaceuticals, San Francisco, CA), phosphorylated tau (PHF-1, 1:500; supplied by Dr. Peter Davies, Albert Einstein Medical School, Bronx, NY), ubiquitin (Dako, Glostrup, Denmark) and phosphorylated α-synuclein (1:10,000; Wako Chemicals USA Inc., Richmond, VA), and phosphorylated TDP-43 (pTDP-43, 1:40,000; Cosmo Bio Inc., Carlsbad, CA). Lewy bodies stage was assessed using a PD staging scale (range: 0, 1-6) [15,16] and the McKeith et al. staging scale [28,29].

**Morphological methods**

In the superior frontal gyrus (SFG) (BA 8,6), cingulate gyrus (CG) (BA 24), and entorhinal cortex (EC) (BA 28), the densities of LB, LN, and LG were counted along strips of tissue oriented parallel to the pia mater, using 250 × 50 µm sample fields arranged contiguously [3]. The sample fields were located both in the upper and lower (laminae V/VI) cortex (minimum n = 32 sample fields in each region), the short edge of the sample field oriented parallel with the pia mater and aligned with guidelines marked on the slide. In the HC, densities of inclusions were measured in sectors CA1 and CA2 (n = 32 fields in total), the short dimension of the sample field being oriented with the edge of the alveus. In the DG (n = 32 fields), the sample fields were aligned to study the molecular and granule cell layers. In the amygdala (n = 16 fields), the fields were arranged across the maximum diameter of the basolateral nucleus, a region with severe α-synuclein pathology in PDD [11]. All distinct α-synuclein-immunoreactive rounded inclusions were counted as LB, LN were thread-like structures often contorted in shape, and small circular dot-like structures were identified as LG [11].

**Data analysis**

To determine patterns of clustering of the LB, LN, and LG, the data were analyzed by spatial pattern analysis [1-3] which was carried out on all brain regions with sufficient density of the pathology, i.e., at least 20 lesions were present along the strip of tissue analyzed. Departure from a random distribution can be measured by calculating the variance/mean (V/M) ratio of the counts of a pathology in contiguous sample fields. If individuals of a pathology are randomly distributed, the number of samples containing 0, 1, 2, 3 ..., n, inclusions should correspond to a Poisson distribution and the V/M ratio should approximate to unity. A V/M ratio less than unity indicates a regular or uniform distribution of individual lesions and greater than unity a clumped or clustered distribution. If a pathology exhibits clustering along the strip of tissue examined, the mean size and distribution of the clusters can be estimated from counts in adjacent sample fields added together successively to provide data for increasing field sizes, e.g., 50 × 250 µm, 100 × 250 µm, 200 × 250 µm etc., up to a size limited by the total length of strip sampled. The V/M ratio is plotted against field size. A V/M peak estimates the presence of regularly-spaced clusters and location of the peak indicates mean cluster size, statistical significance of a V/M peak being tested using the 't' distribution [2,3]. Mean cluster sizes of the LB, LN, and LG were compared among brain regions using a one-way analysis of variance (ANOVA). Relationships between spatial pattern, α-synuclein pathology, and brain region were tested using chi-square (χ²) contingency table tests. Correlations between cluster size of the LB, LN, and LG and patient age, disease duration, and ADNC 'ABC' stages were tested using Pearson’s correlation coefficient (r). As the number of analyses performed was large and without pre-planned hypotheses, p values were adjusted using the Bonferroni correction which suggested that a p value of 0.001 should be used as the critical level to judge significance [5].
Results

Figure 1 shows the typical α-synuclein-immunoreactive pathology in the EC of a case of PDD. Lewy bodies typically occur in small clusters whereas LN and LG are more widespread often developing in larger clusters.

Examples of the spatial pattern analysis are shown in Figure 2. In the upper laminae of the EC (Case A), LB exhibited a V/M peak at a field size of 100 mm suggesting a regular distribution of clusters of LB, 100 mm in diameter, distributed parallel to the pia mater. In the lower laminae of the CG (Case A), LN exhibited a V/M peak at a field size of 400 mm suggesting a regular distribution of clusters 400 mm in diameter, distributed parallel to the pia mater. In the upper laminae of the CG (Case A), there was an increase in V/M of the LG with field size without reaching a peak, suggesting a large cluster of LG of at least 800 mm in diameter.

The frequency of the different spatial patterns exhibited by the LB, LN, and LG in all regions and cases are summarized in Table II. In the majority of regions, the LB, LN, and LG were clustered and most frequently, the clusters were regularly distributed parallel to the tissue boundary. In addition, in a proportion of regions, the LB, LN, and LG were randomly distributed or present in large, non-regularly distributed clusters > 800 mm in diameter. The LB and LN exhibited a similar range of spatial patterns ($\chi^2 = 6.11, p > 0.05$) but the LG were more frequently present in large, non-regularly distributed clusters than the LB ($\chi^2 = 21.04, p < 0.001$) or the LN ($\chi^2 = 9.51, p < 0.05$). No significant differences in the frequencies of spatial patterns were observed in upper compared with lower cortical laminae or in cortical regions compared with CA1/CA2, the dentate gyrus, and basolateral amygdala.

The frequency distribution of cluster sizes of the LB, LN, and LG in cortical regions, viz., SFG, CG, and EC is shown in Table III. The majority of clusters were in the size range of 100-800 mm, significantly fewer gyri having cluster sizes as small as 50 mm or as large as 800 mm. Mean cluster size of the LB, LN, and LG in each brain region is shown in Figure 3. ANOVA suggested that mean cluster size of the LB was significantly less than that of the LN and LG ($F = 7.19, p < 0.001$). There were no statistically significant differences in cluster size among regions.

Correlations among cluster size of the LB, LN, LG and patient age, disease duration, and NIA-AA ‘ABC’ stage are shown in Table IV. Several correlations were present but after Bonferroni adjustment only cluster size of the LN was positively correlated with NFT (‘B’) stage in the lower laminae of the EC ($r = 0.99, p < 0.001$).

Fig. 2. Examples of the spatial patterns exhibited by Lewy bodies (LB), Lewy neurites (LN), and Lewy grains (LG) in a case of Parkinson disease dementia (PDD) (Case A). *Significant variance/mean peaks.

Fig. 1. The α-synuclein pathology of a case of Parkinson disease dementia (PDD) showing Lewy bodies (LB) (star), Lewy neurites (LN) (arrow), and Lewy grains (LG) (arrowhead) in the entorhinal cortex (α-synuclein immunohistochemistry, hematoxylin, bar = 50 mm).
Discussion

In cortical regions in PDD, the LB, LN, and LG were most frequently clustered and in a significant proportion of regions the clusters exhibited a regular distribution parallel to the pia mater. Similar clustering patterns were observed in sectors CA1/CA2 of the HC, DG, and basolateral amygdala. Hence, clustering of the α-synuclein pathology in PDD is similar to that reported previously in various neurodegenerative disorders including the synucleinopathies DLB and MSA [8,10]. Clustering patterns of the α-synuclein pathology in PDD are also similar to that of PrP<sup>sc</sup> deposits in CJD [7,9] in which propagation of PrP<sup>sc</sup> among brain regions is particularly well documented [12,13].

A number of features of the data suggest that α-synuclein may have spread among brain regions via anatomical connections. First, in approximately 60% of cortical gyri, the LB, LN, and LG occurred in...
either small regularly distributed clusters 50-200 mm in diameter or in larger regularly distributed clusters 400-800 mm in diameter. The smaller clusters could reflect an association between the pathology and columns of cells originating either from the midbrain or brain stem nuclei which repeat at 30-40 mm intervals or the commissural and ipsilateral association fibers, which terminate in vertically oriented columns 200-500 mm in width [30]. The larger regularly distributed clusters 400-800 mm in diameter, however, are within the size range of the columns of cells associated with the cortico-cortical pathways [19,22,32]. These cells are themselves clustered and occur in bands regularly distributed along the cortex parallel to the pia mater [19,22]. Individual bands of cells have a mean dimension of approximately 500-1000 mm in width depending on the region, and traverse the laminae in columns; a similar size to the clusters of the LB, LN, and LG is observed in 30% of gyri in PDD. Second, positive correlations between cluster size, disease duration, and NIA-AA ‘A’ or ‘B’ stage in some regions in a region over time or with developing ADNC co-pathology consistent with spread and recruitment of α-synuclein. However, only the correlation between LN and NFT ‘B’ stage in the EC remained significant after Bonferroni adjustment suggesting less convincing evidence for an increasing burden of the pathology as the disease develops. Third, anatomically connected regions exhibited similar regularly distributed clusters, e.g. across the basolateral amygdala and in the CG and EC [17] and in dentate gyrus granule cells and EC (‘perforant path’) [6].

The data suggest a close relationship in PDD between the developing pathology and anatomical pathways which could have resulted from the spread of pathogenic α-synuclein in PDD [14,18,35]. This raises the possibility, first proposed by Braak et al. [21], that a pathogenic agent introduced via ingestion or inhalation, may transfer along axons to basal areas of the brain, the brain stem, and then to the cerebral cortex. α-Synuclein may be the target of this unknown agent causing protein misfolding and subsequent spread of α-synuclein via connecting cells. Hence, olfactory deficits are an early non-motor feature of PD and the pathology could potentially spread via the olfactory bulb and vagal system to the substantia nigra [36]. Further spread into cortical and limbic areas could then occur with subsequent propagation of the pathology among cortical regions.
via the cortico-cortical projections. Such a spread could therefore be an important factor in the development of dementia in PDD.

In conclusion, several features of the spatial patterns of the LB, LN, and LG suggest the anatomical spread of α-synuclein pathology in PDD: (1) the pathology occurred in clusters which were regularly distributed parallel to the tissue boundary, (2) in several regions the estimated cluster size of the LB, LN, and LG was within the size range of cellular columns associated with the cortico-cortical pathways, (3) regularly distributed clusters were present in anatomically connected regions, and (4) the clustering pattern was similar to that of PrPSc deposits in CJD. If this hypothesis is correct, the presence of regularly distributed clusters of the pathology could be a useful indicator of this spread in both human patients and in animal models of the disease.

Acknowledgements

The ‘Movement Disorders Center’ at Washington University School of Medicine in St. Louis is gratefully thanked for providing the PDD cases for this study. In addition, Deborah Carter and Benjamin Vincent of the Betty Martz Laboratory for Neurodegenerative Research are thanked for their expert assistance.

Disclosure

Author reports no conflicts of interest.

References

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