

Neuronal loss and α -synuclein pathology in the superior colliculus and its relationship to visual hallucinations in dementia with Lewy bodies

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1. ABSTRACT

Dementia with Lewy bodies (DLB) patients often experience visual hallucinations, which are related to decreased quality of life for patients and increased caregiver distress. The pathological changes that contribute to visual hallucinations are not known, but several hypotheses implicate deficient attentional processing. The superior colliculus has a role in visual attention, planning eye movements and has been directly implicated in several models of visual hallucinations. Therefore, the present study sought to identify neurodegenerative changes that may contribute to hallucinations in DLB. *Post-mortem* superior colliculus tissue from 13 control, 10 DLB and 10 Alzheimer's disease (AD) cases was evaluated using quantitative neuropathological methods. α -synuclein and tau deposition was more severe in deeper layers of the superior colliculus. DLB cases had neuronal density reductions in the *stratum griseum intermedium*, an important structure in directing attention towards visual targets. In contrast, neuronal density was reduced in all laminae of the superior colliculus in AD. These findings suggest that regions involved in directing attention towards visual targets are subject to neurodegenerative changes in DLB. Considering several hypotheses of visual hallucinations implicate dysfunctional attention towards external stimuli, these findings may provide evidence of pathological changes that contribute to the manifestation of visual hallucinations in DLB.

2. INTRODUCTION

Dementia with Lewy bodies (DLB) is the second most common progressive neurodegenerative dementia after Alzheimer's disease (AD), accounting for approximately 4.2% of all dementia cases (1, 2). DLB is clinically characterized by three core symptoms of fluctuating cognition, parkinsonism and complex visual hallucinations (3). The defining histological feature of DLB, along with Parkinson's disease (PD) and Parkinson's disease dementia (PDD), is the presence of intracytoplasmic α -synuclein-containing inclusions termed Lewy bodies (4). The topographical distribution of Lewy body-related pathology in the respective disorders is thought to underlie the patterns of clinical manifestations (5). Lewy body pathology affects brainstem, limbic and neocortical regions in DLB (6), often in combination with the two hallmark histological features of AD, amyloid- β and tau pathology (3).

Visual attention is more abnormal in DLB than in AD or PD (7), and DLB patients show greater impairment in filtering distracting visual stimuli compared to AD or control patients (8). Deficits in visual attention have been suggested to contribute to the manifestation of complex visual hallucinations in DLB (9, 10). Complex visual hallucinations are common in DLB (11), occurring in 60-80% of cases (12), and typically consist of objects, such as animals, people and faces (13). Visual hallucinations are related to impaired quality of life for DLB patients (14, 15) and contribute to caregiver distress (16), and thus represent an important therapeutic target, yet their pathological etiology is poorly understood.

The superior colliculus has a distinctive laminar structure consisting of seven layers in three major grey matter strata: the *stratum griseum superficiale* (SGS), the *stratum griseum intermedium* (SGI) and the *stratum griseum profundum* (SGP), interspersed with predominantly fibre-rich layers (17). The superior colliculus may be subject to neurodegenerative changes in DLB as increased saccadic latency, which has been reported in DLB (18), has also been observed in non-human primates following chemical inactivation of the superior colliculus (19). The superior colliculus is also involved in attentional functions that are impaired in DLB, such as target selection and the filtering of distracting stimuli (17). One model has directly implicated dysfunction of the dorsal attentional network, with which the superior colliculus is thought to interact, in the manifestation of visual hallucinations in DLB (20). The pathway from

the retina to the inferior pulvinar, routed through the SGS, has also been directly implicated in the manifestation of hallucinations in Lewy body disease (21).

The aim of the present study was to identify whether the superior colliculus is subject to neurodegenerative changes in DLB using stereological measures of cell density and densitometric analysis of neuropathological lesions. Cell density and neuropathological data were then compared to neuropsychological data obtained during life, and the known function of the individual laminae of the superior colliculus, to determine the relationship between neurodegenerative changes and clinical features. DLB cases were compared to aged comparison cases and 'disease comparison' AD cases to elucidate whether degenerative changes occur to the superior colliculus in DLB and their relationship to its distinct clinical manifestation.

3. METHODS

Tissue preparation

Human *post-mortem* tissue was obtained from a convenience sample from the Newcastle Brain Tissue Resource (NBTR) and ethical approval was granted by Newcastle University ethics board and the Joint Ethics Committee of Newcastle and North Tyneside Health Authority (ref: 08/H0906/136). DLB and AD subjects had been part of several prospective clinical studies, and had received detailed clinical assessments according to international consensus guidelines (3, 22) and case note review after death. Cases with psychiatric or neurodegenerative comorbidities were excluded from the present study. Neuropathological assessment was performed according to standardized neuropathological diagnostic procedures (3, 23-26). Clinical and pathological data was collated to establish a clinico-pathological consensus diagnosis. Three groups of cases were included in the present study: 10 DLB cases, 10 AD cases and 13 clinically confirmed aged comparison cases that showed none, or only low, age-associated neurodegenerative pathology at *post-mortem* examination. Case details are contained in Table 1.

At autopsy, the upper midbrain was dissected from the cerebrum at the level of the fourth cranial nerve, along a line from the junction of the mammillary body running posteriorly to the upper part of the superior colliculus (Fig. 1). For stereological analysis, 3x 30 μm adjacent sections were cut and stained with cresyl violet. 6 μm sections were stained with antibodies (KM51 anti- α -synuclein, Leica, UK 1:250; AT8 anti-phosphorylated tau, Autogen, MA, USA, 1:4000; 4G8 anti-amyloid- β , Covance, NJ, USA, 1:15000) using Menarini Menapath polymer detection kits (Menarini, Berkshire, UK).

Stereology

Stereological estimates of neuronal density were made in each of the three prominent grey matter laminae of the superior colliculus: the SGS, the SGI and SGP, based on their cytoarchitecture and laminar organization (27). In cresyl violet sections, neurons were differentiated from glia by the presence of Nissl substance within cytoplasm, a pale nucleus, and a single identifiable nucleolus (28).

Stereological analysis was conducted using a Zeiss AxioVision Z.1 microscope equipped with a motorized stage (Zeiss, Oberkochen, Germany), coupled to a computer with Stereologer software (Bethesda, MA, USA). The rater (D.E.), working together with the senior investigator trained in stereological methodology (A.A.K.), traced an outline around the region of interest (i.e. SGS, SGI or SGP) using a 2.5x objective. Disector frames were placed in a uniform, random arrangement to calculate the density of cells within a defined region (as described previously (28)). Neuronal counts were conducted at 63x oil-immersion objective using the optical disector probe. Glial cell counts were calculated in all laminae in disector frames of 3500 μm^2 , with neuron counts calculated in disector frames of 1900 μm^2 . Section thickness did not vary across disease groups any layer. The mean coefficients of error (CE) for neuronal and glial cell estimates was calculated using the Gundersen-Jensen method (29). The mean coefficient of error values for all stereologically-obtained data showed acceptable levels of accuracy (<0.15) (30).

To evaluate whether neuronal density changes occur as a result of increasing age, *post-mortem* delay or length of time fixed in formalin, correlational analyses were conducted between these variables and neuronal density in each layer of the superior colliculus across all cases. To assess whether neuronal density is altered based on the duration of disease, correlation analyses were conducted between neuronal density in each layer and duration of disease in DLB and AD cases.

Neuropathology

To quantify neuropathological lesions, images of each stratum of the superior colliculus were taken on a Zeiss AxioVision Z.1 microscope using a DsFi1 camera (Nikon, Tokyo, Japan). Stereologer software was used to delineate a region of interest with a 2.5x objective, prior to placement of disector frames in a uniform, random arrangement. This method prevented the introduction of bias by giving every area of the region of interest an equal probability of being sampled for analysis. In all cases, amyloid- β , tau and α -synuclein were measured using a 20x objective. Approximately eight images were taken per lamina per case within the disector frames and analyzed using ImagePro Plus v.4.1 image analysis system (Media Cybernetics, Bethesda, MA, USA). Using previously published techniques (31) the mean percentage area of

immunopositivity was determined by standardizing red-green-blue (RGB) thresholds per antibody and applying to all sections per case. Each case thus had a mean value generated per antibody for each stratum.

The percentage area immunoreactive for α -synuclein, tau and amyloid- β was compared across disease groups to evaluate the vulnerability of the superior colliculus to neurodegenerative pathology in DLB, AD and comparison cases. Correlational analyses were conducted between neuropathological and stereological data to evaluate the relationship between neuropathology and neuronal density in each disease group.

Clinico-pathological correlation

DLB patients had been recruited for clinical research studies, during which some had received serial assessments of visual hallucination severity and frequency at frequent intervals until death (13, 32). Due to the hypothesized role of the superior colliculus in the manifestation of visual hallucinations in Lewy body disease (21), neuronal density and pathological burden were correlated with final neuropsychiatric inventory hallucinations subscale score (NPI [hall]) in all layers of the superior colliculus (33) in DLB cases, where available. Final NPI (hall) scores were available for 8/10 DLB cases. The mean interval from final NPI assessment to death was 7.81 ± 3.45 months. The relationship between visual hallucinations and the superior colliculus was not explored in AD as visual hallucinations are not a core feature of AD, and were less frequently assessed. Additionally, as a separate disease entity, it is possible that visual hallucinations in AD, when present, have a different underlying pathogenesis to that in DLB.

Statistics

Inspection of Q-Q plots and Shapiro-Wilk tests suggested that demographic, stereological and neuropathological data were either not normally distributed or did not have homogeneity of variance. Therefore, Kruskal-Wallis (KW) tests with post-hoc Mann-Whitney U tests were employed for these data. Due to the relatively small

sample size, corrections for multiple comparisons were not applied. However, effect sizes were reported using Cohen's *d*. For comparisons between different laminae, Friedman tests with post-hoc Wilcoxon signed-ranks tests were performed.

4. RESULTS

Demographics

No significant differences were found between groups in age at death (KW $\chi^2=2.461$, $df=2$, $p=0.292$), *post-mortem* delay (KW $\chi^2=2.708$, $df=2$, $p=0.258$) or length of time fixed in formalin (KW $\chi^2=0.472$, $df=2$, $p=0.790$; Table 1).

Stereology

In the SGS, there was a significant main effect of diagnosis on neuronal density (KW $\chi^2=6.579$, $df=2$, $p=0.037$). No significant difference was found between comparison cases and DLB. AD cases (mean[x100]=0.0054 [95% CI 0.0047-0.0061]) had reduced neuronal density compared to comparison cases (mean[x100]=0.0067 [95% CI 0.0059-0.0076]; $U=27.000$, $p=0.018$, $d=1.014$). There was no significant difference in neuronal density between DLB and AD in the SGS. There was no significant main effect of diagnosis on glial density in the SGS (Fig. 2).

In the SGI, there was a significant main effect of diagnosis on neuronal density (KW $\chi^2=13.046$, $df=2$, $p=0.001$). DLB (mean[x100]=0.0030 [95% CI 0.0025-0.0034]; $U=18.500$, $p=0.003$, $d=1.08$), and AD (mean[x100]=0.0029 [95% CI 0.0026-0.0032]; $U=13.5$, $p=0.001$, $d=1.24$) had reduced neuronal density compared to comparison cases (mean[x100]=0.0036 [95% CI 0.0033-0.0038]). There was no significant difference in neuronal density between DLB and AD in the SGI. There was no significant main effect of diagnosis on glial density in the SGI (Fig. 2).

In the SGP, there was a significant main effect of diagnosis on neuronal density (KW $\chi^2=6.600$, $df=2$, $p=0.037$). No significant difference was found between comparison cases and DLB. AD cases (mean[x100]=0.0028 [95% CI 0.0025-0.0031]) had reduced neuronal density compared to comparison cases (mean[x100]=0.0036 [95% CI 0.0031-0.0040]; $U=27.000$, $p=0.018$, $d=1.12$). There was no significant difference in neuronal density between DLB and AD in the SGP. There was no significant main effect of diagnosis on glial density in the SGP (Fig. 2).

In DLB cases, duration of disease was significantly negatively correlated with neuronal density in the SGI ($r_s=-0.726$, $N=10$, $p=0.018$). However, no significant correlations

were found between duration of disease and neuronal density in the SGS and SGP. In AD, duration of disease was not significantly correlated with neuronal density in any layer.

Neuropathology

Comparisons of neuropathology between groups are detailed in Table 2.

In DLB cases, there was a significant difference in the degree of α -synuclein pathology across the three layers (Friedman $\chi^2=12.600$, $df=2$, $p=0.002$; Fig. 3A-C). The SGI (mean=0.0268 [95% CI 0.0180-0.0356]; Wilcoxon $Z=2.803$, $p=0.005$, $d=0.85$), and the SGP (mean=0.0317 [95% CI 0.0108-0.0525]; Wilcoxon $Z=2.497$, $p=0.013$, $d=1.09$), had a significantly greater burden of α -synuclein pathology than the SGS (mean=0.0090 [95% CI 0.0025-0.0155]). There was no significant difference in the burden of α -synuclein pathology between the SGI and SGP. There were no significant correlations between α -synuclein pathology and neuronal density in any experimental group.

Across DLB, AD and comparison cases, there was a significant difference in the degree of tau pathology between the three layers (Friedman $\chi^2=26.375$, $p<0.001$; Fig. 3D-F). The SGI (mean=0.0929 [95% CI 0.0074-0.1783]; Wilcoxon $Z=4.330$, $p<0.001$, $d=0.32$) and SGP (mean=0.1241 [95% CI 0.0130-0.2352]; Wilcoxon $Z=4.031$, $p<0.001$, $d=0.46$) had a significantly greater burden of tau pathology than the SGS (mean=0.0207 [95% CI 0.0038-0.0376]). There was no significant difference in the burden of tau pathology between the SGI and SGP. Tau pathology was significantly negatively correlated with neuronal density in the SGI in all cases ($r_s=-0.404$, $N=33$, $p=0.022$) and SGP ($r_s=-0.451$, $N=33$, $p=0.009$). However, there was no significant relationship between tau pathology and neuronal density in the SGS.

Across DLB, AD and comparison cases, there was no significant difference in amyloid- β expression across the three layers of the superior colliculus (Fig. 3G-I). There were no significant correlations between amyloid- β pathology and neuronal density in any experimental group.

Clinico-pathological relationships

There were no significant correlations between age at death, *post-mortem* delay and fixation time in formalin, and neuronal density in any layer of the superior colliculus.

In DLB cases, NPI (hall) score was significantly positively correlated with neuronal density in the SGS ($r_s=0.874$, $N=8$, $p=0.005$). No other stereological or pathological variables were correlated with NPI (hall) score in DLB cases.

5. DISCUSSION

This study demonstrates reductions in neuronal density in the SGI in DLB, compared to neuronal loss in all laminae in AD. α -synuclein pathology was not related to neuronal density in any layer in DLB, but tau was inversely related to neuronal density in the SGI and SGP. There was no relationship between α -synuclein pathology and hallucination severity and frequency, as assessed by NPI (hall). However, neuronal density in the SGS was positively correlated with the severity and frequency of visual hallucinations in DLB. Distinct topographical patterns of deposition were observed for α -synuclein and tau pathology, with the SGI and SGP affected more severely than the SGS.

The SGS receives retinal input (34) and projects to the medial temporal visual area (area V5/MT) through the inferior pulvinar (35, 36) in a pathway thought to be involved in the non-conscious perception of motion (37). In contrast, the SGI and SGP receive inputs from wide-ranging cortical and sub-cortical regions, such as motor and association sensory cortices, the locus ceruleus and dorsal raphe (38), and have an important role in the selection of visual targets (39) and in directing the resulting motor output (40).

Within the superior colliculus, neuropathological lesions occurred in a stereotypical manner, with higher levels of α -synuclein and tau found in the SGI and SGP compared to the SGS. This may reflect the SGI and SGP receiving inputs from the dorsal raphe, locus ceruleus and pedunculopontine nucleus, which are vulnerable to the accumulation of Lewy body and tau pathology (41, 42). In contrast, the SGS receives predominant innervation from the retina and primary visual cortex (17), which do not appear to accumulate significant Lewy body pathology in DLB, and develop tau pathology only at the final stage of AD pathology (43-46). As tau and α -synuclein may spread throughout the brain in a manner reminiscent of prion protein, these distinct patterns of deposition may reflect greater connectivity with areas that are vulnerable to early neuropathological lesion formation (47). However, a neuropathological study of the superior colliculus has reported greater tau pathology in the SGI and SGP in individuals with chronic traumatic encephalopathy, indicating that these laminae may have an intrinsic vulnerability to tau pathology (48).

The present study has demonstrated that neuronal loss in the superior colliculus only occurs in the SGI in the DLB cases included in this study. This was in contrast to AD, where neuronal density reductions were found in all laminae of the superior colliculus. DLB had significantly increased tau in the SGI, but not in other layers of the superior colliculus. As tau was negatively correlated with neuronal density in the SGI and SGP across all cases, this may indicate that tau is driving neuronal density reductions in DLB. The greater levels of tau pathology in AD cases may similarly cause the reductions in neuronal density seen in all laminae of the superior colliculus.

Previous studies in the LGN and primary visual cortex (31, 44) have not demonstrated neuronal loss in DLB. In contrast, neuronal loss has been identified in the lateral pulvinar (49) and in the SGI of the superior colliculus in DLB. One model of visual hallucinations has suggested that DLB patients have difficulty engaging the dorsal attentional network to focus attention on ambiguous stimuli, instead relying on the self-referential default mode network (20). As the lateral pulvinar (50) and SGI (17) are implicated in visual attention and target selection, respectively, it is tempting to speculate that neuronal losses in these regions could impair directed attention toward visual targets. However, visual attentional measures were not conducted on the present cases, so it is not possible to confirm whether these patients were impaired in these faculties, or whether this related to more severe visual hallucinations. Additionally, visual hallucinations in DLB are unlikely to result purely from neuronal loss in the SGI and these changes likely act in concert with degenerative changes elsewhere in the visual system to elicit visual hallucinations.

Visual dysfunction, including hallucinations, in Lewy body disease have been linked to dysfunctional pathways involved in blindsight, the phenomenon whereby a blind individual may react to moving or emotional visual stimuli (21). These pathways implicate the SGS, a region that has been found in the present study to be preserved in individuals with more severe and frequent visual hallucinations. This may indicate that dysfunction of this region, or the pathway through it, is crucial for the occurrence of visual hallucinations. The present study was conducted on a relatively small sample, so it is difficult to draw strong conclusions about the relationship between neuronal number in the SGS and visual hallucinations.

This study was limited by the fact that the superior colliculus was sampled on three adjacent slides, rather than serially through the entire z-axis. As a result, volume and total neuronal number could not be estimated. This was due to the superior colliculus being routinely dissected on two different planes (coronal and axial) and the inherent value of upper midbrain tissue. Few cases were available that had NPI data on visual hallucinations obtained during life and this impacted the number of cases included in the study. However, the use of a comparator group that do not typically manifest visual hallucinations suggests that the reported findings may be specific to DLB.

In summary, the present study has demonstrated that the superior colliculus is subject to neurodegenerative changes in DLB. Specifically, neuronal loss was only found in the SGI in DLB. In contrast, AD cases had neuronal loss in all layers. As the SGI is important in directing attention and aiding visual target selection, the present results may indicate dysfunction in these functions in DLB. As dysfunctional visual attention and target selection have been related to visual hallucinations in DLB, the present results may provide evidence of neuropathological changes that contribute to the manifestation of visual hallucinations in DLB. However, these changes are unlikely to induce visual hallucinations on their own, thus continued study of the visual system is necessary to further understand the pathological changes that cause visual hallucinations in DLB.

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Figure legends

Fig. 1: Upper midbrain section stained with Loyez's hemotoxylin. The superior colliculus is circled.

Fig. 2: Stereological analysis of neuronal density. Neuronal and glial cell density across experimental groups in each lamina. * $p < 0.05$.

Fig. 3: Images of pathology in a DLB case. α -synuclein pathology is less severe in the SGS (A) than the SGI (B) and SGP (C). Tau pathology is minimal throughout but is less severe in the SGS (D) than in the SGI (E) and SGP (F). Amyloid- β is observed in the SGS (G), SGI (H) and SGP (I) and shows less regional specificity than α -synuclein and tau. Scale bar = 50

