Molecular changes in the absence of severe pathology in the pulvinar in dementia with Lewy bodies.

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ABSTRACT

Background

Dementia with Lewy bodies is characterized by transient clinical features, including fluctuating cognition and visual hallucinations, implicating dysfunction of cerebral hub regions, such as the pulvinar nuclei of the thalamus. However, the pulvinar is typically only mildly affected by Lewy body pathology in dementia with Lewy bodies, suggesting additional factors may account for its proposed dysfunction.

Methods

We conducted a comprehensive analysis of *post-mortem* pulvinar tissue using whole-transcriptome RNA sequencing, protein expression analysis and histological evaluation.

Results

We identified 321 transcripts as significantly different between dementia with Lewy bodies cases and neurologically normal controls, with gene ontology pathway analysis suggesting enrichment of transcripts related to synapses and positive regulation of immune function. At the protein level, proteins related to synaptic efficiency were decreased, whilst general synaptic markers remained intact. Analysis of glial sub-populations revealed astrogliosis without activated microglia, which was associated with synaptic changes but not neurodegenerative pathology.

Discussion

These results indicate that the pulvinar, a region with relatively low Lewy body pathological burden, manifests changes at the molecular level which differ from previous reports in a more severely affected region. We speculate that these alterations result from neurodegenerative changes in regions connected to the pulvinar, and likely contribute to a variety of cognitive changes resulting from decreased cortical synchrony in dementia with Lewy bodies.

INTRODUCTION

Dementia with Lewy bodies (DLB) is thought to be the second most common form of neurodegenerative dementia after Alzheimer's disease (AD) (1). Clinically, DLB is marked by four core symptoms of fluctuating cognition, parkinsonism, visual hallucinations and rapid eye movement sleep behavior disorder, against the backdrop of global cognitive decline (2). Pathologically, DLB is characterized by pathological aggregates of α-synuclein in nerve cell bodies and nerve cells processes termed Lewy bodies and Lewy neurites, respectively (3). However, varying degrees of AD-type pathology, consisting of extracellular amyloid-β plaques and intraneuronal tangles of abnormally hyperphosphorylated tau, are frequent concomitant features (4, 5).

Visuo-perceptual and attentional functions are impaired in DLB (6-8), and may promote the occurrence of visual hallucinations (9-12). The pulvinar contributes to visuo-perceptual and attentional functions (13), has reciprocal connectivity with widespread cortical regions (14), and is a putative 'hub' that coordinates neural activity across the cortex (15). Dysfunction of highly interconnected hubs has been postulated as important in eliciting the clinical manifestation of neurodegenerative disorders, including DLB, by diminishing network coherence and coordinated neural activity (16). Whilst most research on network connectivity in neurodegenerative disorders has focused on AD (17), connectivity is decreased to a greater degree in DLB compared to AD, with particular impairments in long-distance connections (18).

Metabolic deficits (19) and increased tissue diffusivity (20) have previously been reported in the pulvinar in DLB. We have previously reported neuronal loss in the pulvinar, which may promote attentional dysfunction and visual hallucinations in DLB (21). However, Lewy body pathology is relatively mild in the pulvinar (22) and the sub-regions most severely affected by α-synuclein aggregation did not show neuronal loss (21). Therefore, it is difficult to relate the myriad changes described previously in the pulvinar with the manifest burden of α-synuclein pathology. On that basis, we have investigated differential gene expression with whole-transcriptome RNA sequencing (RNA-seq), protein quantification assays and histological analysis to evaluate changes to the pulvinar which may be relevant to the clinical features of DLB.

METHODS

Tissue preparation

All tissue was obtained from Newcastle Brain Tissue Resource (NBTR), a UK Human Tissue Authority-approved research tissue repository, 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 cases had been part of prospective clinical studies, and had received detailed clinical assessments during life and case note review after death. All cases had consented to the use of their brain tissue for research purposes. Neuropathological assessment was conducted according to standardized neuropathological diagnostic procedures (4, 23-26). Clinical and pathological data was collated to establish a clinico-pathological consensus diagnosis. The present study included cases with a clinical diagnosis of DLB confirmed by neuropathological *post-mortem* assessment. DLB cases were compared to aged individuals with an absence of neurological features *intra vitam* low age-associated neurodegenerative pathology. Demographic information is provided in Supplementary Tables 1 and 2.

At autopsy, tissue from the left hemisphere was cut into 1 cm thick coronal sections and rapidly frozen at -80°C between copper blocks. The pulvinar was identified by its location in the posterior pole of the thalamus from which approximately 50 mg of tissue was dissected with a cooled scalpel (27). Frozen tissue was obtained from a cohort of 15 control and 14 DLB cases (Supplementary Table 1).

The right hemisphere was fixed in 10% formalin and dissected into blocks for neuropathological assessment. 10 µm sections were taken from the pulvinar at the level of the posterior aspect of the lateral geniculate nucleus and the amygdala and stained with antibodies against a range of protein targets using Menarini Menapath Polymer detection kits (Menarini, Berkshire, UK) and counterstained with haematoxylin. Fixed pulvinar tissue was obtained from a cohort of 14 controls and 14 DLB cases (Supplementary Table 2).

RNA isolation and sequencing

Frozen tissue was placed in 5-10 volumes of pre-cooled RNA*later* solution (Ambion, Warrington, UK) and stored at -80°C. Tissue was removed from RNA*later* and rapidly homogenized in TRI-reagent (Ambion) and stored at -80°C. RNA was extracted using a spin column method, as per manufacturer's instructions (Ribopure, Ambion), and 1 µg of RNA was DNase-treated (Turbo-DNase free, Ambion). The RNA concentration was determined using a Nanodrop ND 1000 Spectrophotometer (Nanodrop Technologies) and RNA integrity number (RIN) examined with an Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Stockport, UK).

RNA-seq libraries were prepared using TruSeq Ribo Zero Gold kits (Illumina, CA, USA). Clustering was performed with 10 nM libraries pooled in groups of six libraries per lane of each flow cell. We then sequenced 200 bp paired-end libraries on a HiSeq2500 sequencer. Sequence reads were aligned using Salmon []. Genes with low expression (row mean counts for <1) were removed, then differential expression was estimated using DESEQ2 (28) using the following model to correct for biological correlates:

Expression ~ Age + Gender + Post-mortem duration + Disease

Within DESEQ2, p-values for differential expression from Wald tests were corrected for multiple testing using the Benjamini-Hochberg false discovery rate approach, with significant results reported at α=0.05. Gene ontology (GO) enrichment was performed using gProfileR (29).

Transcriptomic changes were evaluated at the protein level using western blot analysis (Supplementary Protocol 1).

Microscopy

To quantify glial sub-populations and neuropathological lesions in the pulvinar in a separate cohort of cases and α-synuclein pathology in the amygdala of the cases used for the transcriptomic study, images were taken on a Zeiss AxioVision Z.1 microscope using a DsFi1 camera (Nikon, Japan). As detailed previously (21, 30), 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. Disector frame sizes were determined based on the size of the measured particles and their distribution across the region of interest. In all cases, amyloid-β (4G8 anti-amyloid-β, Covance, NJ, USA, 1:15000) was analyzed using 10x objective and α-synuclein (5G4 anti-α-synuclein, Analytik Jena, Germany, 1:4500) and tau (AT8 anti-tau, Autogen, MA, USA, 1:4000); the microglial markers HLA-DP/DQ/DR (CR3/43, Dako, Denmark, 1:1000), CD74 (LN-2, Santa Cruz, USA, 1:500) and Iba1 (Wako, Japan, 1:1000); and the astrocytic markers GFAP (Z0334, Dako, Denmark, 1:10000) and ALDH1L1 (N103/39, Millipore, MA, USA, 1:7500) were measured using 20x objective.

We determined the percentage area occupied by individual antibodies by analyzing images by determining red-green-blue (RGB) thresholds using ImagePro Plus v.4.1 image analysis system (Media Cybernetics, Bethesda, MA, USA). Size restriction was used with the 4G8 antibody to ensure intracellular amyloid-β was not included in the analysis. In addition to quantitative analysis, we qualitatively assessed Iba1 morphology as described previously (31). We also qualitatively determined the presence of Alzheimer Type II astrocytes, the histopathological hallmark of manganism and hepatic encephalopathy (32), as their presence was noted in a substantial number of cases.

These findings were correlated with densitometric analyses of neuropathological lesion burden to evaluate whether neuroglial marker expression was related to pathological protein deposition. A sub-set of cases used for histological analysis (8/14 control; 8/14 DLB) had been assessed as part of a previous stereological study of the pulvinar (21). Therefore, we additionally included stereological determination of total neuronal number within these analyses.

RESULTS

Demographic data

Demographic data for the RNA-seq and protein expression analysis cohort is shown in Supplementary Table 1. There was no significant difference between groups in age at death (t=0.18, df=22, p=0.862), *post-mortem* interval (t=0.17, df=22, p=0.863), and, where available, tissue pH (t=0.60, df=15, p=0.555). There was no significant difference in the proportion of males relative to females between DLB and control (χ^2=2.10, df=1, p=0.148). Braak NFT stage was significantly higher in DLB compared to control (t=3.85, df=19, p=0.001).

Demographic data for the histological analysis cohort is shown in Supplementary Table 2. There was no significant difference between groups in age at death (t=0.0.23, df=26, p=0.982) or *post-mortem* interval (t=1.23, df=26, p=0.217). There was no significant difference in the proportion of males relative to females between DLB and control (χ^2=0.57, df=1, p=0.706). Braak NFT was significantly higher in DLB cases compared to controls (t=3.88, df=26, p=0.001).

Nomination of differential pulvinar gene expression between DLB and controls by RNA sequencing

Our RNA-seq analysis revealed a partial separation between DLB cases and controls in overall gene expression (Fig. 1). Quality control data is included in supplementary QC file. From this analysis, we nominated 321 transcripts significantly different between controls and DLB cases after correction for multiple testing. Subsequently, GO enrichment analysis demonstrated several pathways were enriched in DLB cases compared to control (Table 1). We focused on genes related to synapses (GO:0045202, p=1.75E-25) and positive regulation of immune system process (GO:0002684, p=7.75E-22).

Validation of synaptic and immune proteins by western blot analysis

Analysis of protein expression using western blot analysis of general pre-synaptic markers demonstrated significantly lower expression of synaptophysin (U=30, p=0.015), NSF (U=25.5, p=0.006) and dynamin (U=37.5, p=0.047) in DLB compared to control (Figure 2). This was consistent with RNA-seq data, which demonstrated significantly lower expression of SYP (p=0.01), NSF (p=0.01) and DNM1 (p=0.03). However, no significant differences were found in STX1A, SNAP25, SV2B or GAP43, despite significantly lower expression at the mRNA level.

Analysis of protein expression using western blot analysis of general post-synaptic markers identified significantly lower expression of the dendritic marker MAP2 (U=35, p=0.034) in DLB compared to control (Figure 2), consistent with lower MAP2 mRNA (p=0.04). The excitatory synaptic markers PSD-93 (U=25.5, p=0.011) and PSD-95 (U=27, p=0.009) were also lower in DLB compared to control (Figure 2), consistent with reductions in DLG3 ($p<0.01$) and DLG4 ($p<0.01$) mRNA.

The inhibitory synaptic marker GABARAP (U=37, p=0.046) was significantly reduced in DLB compared to control (Figure 2), consistent with a reduction in GABARAP mRNA (p=0.04). However, protein levels of the inhibitory post-synaptic marker gephyrin were not significantly lower in DLB compared to control, despite being lower at the mRNA level. The GABA-ergic neuron marker GAD67 was lower in DLB compared to control on western blot (U=32, p=0.022), and also at the mRNA level (p=0.02; Figure 2).

Analyses of CHI3L1, a positive regulator of immune system process and pro-inflammatory marker (33), demonstrated significantly higher protein levels (U=35, p=0.034; Figure 3). The astrocytic marker GFAP was also higher in DLB relative to control (U=37, p=0.046; Figure 3). HSPA1B was significantly increased in DLB compared to control (U=22, p=0.003). However, SERPINH1/HSP47 and HSPA1A were not significantly different in DLB compared to control cases (Figure 3), despite showing differences for the same marker in RNA-seq.

Microscopy

As RNA-seq demonstrated an increase in transcripts related to positive regulation of immune system process, we histologically assessed markers of microglia and

astrocytes, the resident immune cells of the brain, in a separate cohort of DLB and control cases. We assessed the expression of the cytotoxic M1 microglial markers CD74 and HLA-DP/DQ/DR, the general microglial marker Iba1 and the astrocytic markers ALDH1L1 and GFAP in the pulvinar of DLB cases compared to control. We also assessed α-synuclein, amyloid-β and tau expression to evaluate whether immune cell expression was related to the presence of neurodegenerative pathologies.

The observed Lewy body pathology was greater than that previously reported in another study of the pulvinar in DLB, which described an absence of Lewy bodies but sparse neuritic pathology (22). This discrepancy may be the result of our use of the 5G4 antibody, which is reported to show more widespread α-synuclein pathology (34). Nevertheless, Lewy bodies were not frequently encountered within the pulvinar of most cases, with Lewy body burden typically corresponding to absent or mild deposition under previously described semi-quantitative assessment methods (4). However, we noted an abundance of α-synuclein immunoreactive dots and occasional fine threads, as noted previously with the 5G4 antibody (35).

α-synuclein ($U=0$, $p<0.001$), amyloid-β ($U=39$, $p=0.006$) and tau ($U=37$, $p=0.004$) were higher in the pulvinar of DLB cases compared to those of controls (Fig. 4). Although AIF1 mRNA was significantly elevated in the DLB pulvinar on RNA-seq ($p=0.003$), its protein product Iba1 was not increased on histological analysis (Supplementary Figure 1). Similarly, CD74 mRNA was significantly higher in DLB ($p=0.02$) but was not different on histological analysis (Supplementary Figure 1). Although some specific sub-types of HLA-D were significantly increased at the mRNA level, there was no significant difference in expression of HLA-DP/DQ/DR on histological analysis (Supplementary Figure 1). The astrocytic marker ALDH1L1 was not significantly different on RNA-seq or histological analysis between DLB and controls. However, GFAP was significantly higher in DLB cases compared to controls at the mRNA level ($p=0.001$) and on histological analysis ($U=18$, $p=0.001$; Supplementary Figure 1).

A range of different microglial morphologies were observed across cases and within experimental groups (Supplementary Figure 2). Possibly as a result of the considerable heterogeneity in morphologies within groups, no morphology was

significantly associated with either experimental group (χ^2=4.5, p=0.214; Supplementary Figure 3). Furthermore, individual microglial morphologies were not associated with any histopathological or glial marker. Alzheimer type-II astrocytes were not more frequently encountered in DLB cases compared to control (χ^2=2.8, p=0.104; Supplementary Figure 3).

Relationship between synaptic loss and neuropathological changes

To evaluate whether synaptic loss corresponded to neuropathological changes in a region projecting to the pulvinar, we quantified the burden of α-synuclein pathology in the amygdala, a region connected to the pulvinar through a pathway reported to be dysfunctional in DLB (20). Of the nine synaptic markers significantly reduced in DLB compared to control only PSD-93 was significantly negatively correlated with α-synuclein burden in the amygdala (r_s=-0.729, p=0.017; Supplementary Figure 4).

Relationship between astrocytic increases and neuropathological, stereological and synaptic changes

After identifying an increase in GFAP in DLB compared to control, we next evaluated the relation of this marker to the presence of neuropathological lesions, neuronal loss and synaptic changes. To prevent spurious correlations being identified due to group differences, DLB cases were analyzed separately from controls. The histological expression of GFAP was not significantly related to amyloid-β, tau or α-synuclein in DLB cases. Within the sub-set of cases assessed using stereological determination of neuronal number (8/14) as reported previously (21), GFAP was not related to neuronal number.

Employing two distinct cohorts of cases for transcriptomic and histological analyses limited our ability to compare histologically assessed glial markers and synaptic markers assessed with western blot. Therefore, we also assessed GFAP using western blot analysis to investigate whether GFAP expression was related to synaptic changes in DLB. GFAP (50 kDa) was significantly negatively correlated with

synaptophysin (r_s=-0.621, p=0.041), dynamin (r_s=-0.655, p=0.029), GABARAP (r_s=-0.673, p=0.023), and GAP43 (r_s=-0.627, p=0.039) in DLB cases.

Clinico-pathological correlations

A sub-set of DLB cases (9/14) used for histological analysis had been subject to neuropsychological evaluation *intra vitam*. As detailed previously (30), these individuals had been assessed using the hallucinations subscale of the Neuropsychiatric Inventory (NPI) within two years prior to death (36). Comparison of NPI hallucinations score with neuropathological markers and GFAP demonstrated a significant positive correlation only between tau burden and NPI hallucination subscale score (r_s=0.701, p=0.035). There were no significant correlations between NPI hallucinations subscale and any other variable.

DISCUSSION

Using a transcriptomic approach, the present study has demonstrated synaptic changes and astrogliosis in the pulvinar in DLB. Notably, these findings occurred in a region that typically manifests relatively mild α-synuclein deposition yet is postulated to play a central role in the cognitive profile of DLB. The reported changes differ markedly from a previous study that employed RNA-seq in the cingulate gyrus, a region with more severe α-synuclein pathology (2), and which reported genes involved in neurogenesis and myelination enriched in DLB compared to control (37).

The reported synaptic changes indicate lower expression levels of pre-synaptic proteins such as synaptophysin and NSF which support efficient turnover of vesicles following exocytotic events (38). In contrast, we found preservation of proteins necessary for vesicular exocytosis, such as SNAP25 (39), STX1A (40), and SV2B (41). Despite the interaction of α-synuclein with synaptic proteins, previous studies have not consistently demonstrated significantly lower levels of pre-synaptic markers in DLB (42).

The role of glia in DLB has been a matter of controversy and debate, with conflicting reports in the literature. Microglial activation is induced by aggregated α-synuclein *in vitro* (43), though *post-mortem* studies have reported inconsistent findings (31, 44-46). Despite RNA-seq demonstrating enrichment of transcripts related to positive regulation of immune system processes, we found no evidence of such changes at the protein level. Therefore, our data favor the view that microglia-mediated neuroinflammatory processes are not an important factor in the reported synaptic changes. However, it is impossible to exclude the possibility that an acute inflammatory response occurred earlier in the disease process but was undetectable in terminal stages.

As GFAP immunoreactivity did not correlate with any pathological lesion or neuronal loss, astrogliosis does not seem to be a response to neurodegenerative lesions within the pulvinar. It is noteworthy that astrogliosis was not accompanied by microgliosis and thus does not appear to signify a neuroinflammatory state. Considering the negative relationship between reactive astrogliosis and several synaptic markers, we speculate that reactive astrogliosis may be a response to synaptic dysfunction, with the aim of supporting synaptic transmission. Further

studies are warranted to evaluate the role of astrocytes in Lewy body diseases, and whether they have a protective or degenerative function. Elucidating the role of astrocytic sub-populations in neurodegenerative disorders may identify novel therapeutic targets to augment protective functions or attenuate degenerative processes.

The role of the pulvinar as a 'hub' modulating cortico-cortical activity may suggest that the present findings are the neuropathological substrate of desynchronous network coherence in DLB. The pulvinar exerts a powerful influence on cortical activity based on attentional demands (47) meaning its dysfunction likely impacts attention-mediated cortical functions. Attention is deficient in DLB (48, 49), and has been implicated in visual hallucinations and fluctuating cognition (11, 48). The search for the neuropathological substrates of symptoms such as visual hallucinations and cognitive fluctuations is impeded by the inherent difficulty in attributing a transient feature to a permanent neuropathological change. However, dysfunction of structures regulating cortical functioning on the basis of attention may be more likely to contribute to transient features of neurodegenerative diseases.

The reported findings are within a region with relatively low levels of Lewy body pathology and differ from those reported in a more severely affected region, the cingulate gyrus (37). These findings indicate important molecular changes, in addition to previously reported neuronal loss (21), independent of the severity of local neuropathological changes. Although we noted a relationship between tau pathology in the pulvinar and the frequency and severity of visual hallucinations *intra vitam*, the overall levels of tau were very low in the pulvinar in DLB. Furthermore, these findings are hard to reconcile with our previous report of higher tau burdens in the pulvinar of Alzheimer's disease cases without visual hallucinations compared to DLB (50). The tau burden in the pulvinar may be a proxy measure of global tau burden, which has been previously reported to influence the clinical phenotype of DLB (51).

As the pulvinar is highly interconnected with numerous cortical and sub-cortical areas, one may speculate that the reported findings are a downstream result of neuropathological changes to regions connected to the pulvinar. We identified a strong negative correlation between PSD-93 and α-synuclein burden in the

amygdala, a region connected to the pulvinar. Whilst a similar relationship was not found with other synaptic markers, the pulvinar as a 'hub' region has widespread connectivity across the cortex (13) and a systematic evaluation of the many regions connected to it was beyond the scope of this study. Molecular changes in 'preserved' regions as a result of neuropathological changes elsewhere may be particularly relevant to the aetiopathology of Lewy body diseases, considering the relatively selective topography of α-synuclein deposition (52). Therefore, relative preservation of brain structures may have important implications for the clinical phenotype of DLB and studies focusing only on regions with severe α-synuclein deposition may miss pathological alterations relevant to the clinical phenotype of Lewy body disease.

In summary, we have identified changes on the molecular level in the pulvinar, a region with relatively low levels of Lewy body pathology, but that is thought to have an important influence upon the cognitive phenotype of DLB (13). One may speculate that the reported synaptic and astroglial changes are a downstream effect of neurodegenerative changes elsewhere and suggest that the absence of a significant local pathological burden should not be assumed to indicate functional preservation.

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AUTHOR CONTRIBUTIONS

Preparation of tissue homogenates and RNA isolation: DE and CMM

RNA sequencing and bioinformatics: JD, AK and MRC

Cutting of histological sections: AAK and DE

Neuropathological diagnosis of cases: JA

SDS-PAGE and western blot analysis, and staining and analysis of histological sections: DE

Interpretation of clinical notes: AJT, JPT and IGM

Preparation of manuscript: DE

Critical revision of manuscript for important intellectual content: AJT, AAK, PSH, JPT, IGM, JA, MRC and CMM

Concept and funding of study: CMM, AAK and AJT

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