

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

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1 ABSTRACT

2 *Background*

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

8 *Methods*

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

12 *Results*

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

20 *Discussion*

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

27

28

## 1 INTRODUCTION

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

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

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

## 1 METHODS

### 2 Tissue preparation

3 All tissue was obtained from Newcastle Brain Tissue Resource (NBTR), a UK  
4 Human Tissue Authority-approved research tissue repository, and ethical approval  
5 was granted by Newcastle University Ethics Board and the Joint Ethics Committee of  
6 Newcastle and North Tyneside Health Authority (ref: 08/H0906/136). DLB cases had  
7 been part of prospective clinical studies, and had received detailed clinical  
8 assessments during life and case note review after death. All cases had consented  
9 to the use of their brain tissue for research purposes. Neuropathological assessment  
10 was conducted according to standardized neuropathological diagnostic procedures  
11 (4, 23-26). Clinical and pathological data was collated to establish a clinico-  
12 pathological consensus diagnosis. The present study included cases with a clinical  
13 diagnosis of DLB confirmed by neuropathological *post-mortem* assessment. DLB  
14 cases were compared to aged individuals with an absence of neurological features  
15 *intra vitam* low age-associated neurodegenerative pathology. Demographic  
16 information is provided in Supplementary Tables 1 and 2.

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

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

29

### 30 RNA isolation and sequencing

1 Frozen tissue was placed in 5-10 volumes of pre-cooled RNA*later* solution (Ambion,  
2 Warrington, UK) and stored at -80°C. Tissue was removed from RNA*later* and  
3 rapidly homogenized in TRI-reagent (Ambion) and stored at -80°C. RNA was  
4 extracted using a spin column method, as per manufacturer's instructions (Ribopure,  
5 Ambion), and 1 µg of RNA was DNase-treated (Turbo-DNase free, Ambion). The  
6 RNA concentration was determined using a Nanodrop ND 1000 Spectrophotometer  
7 (Nanodrop Technologies) and RNA integrity number (RIN) examined with an Agilent  
8 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Stockport, UK).

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

16  $\text{Expression} \sim \text{Age} + \text{Gender} + \text{Post-mortem duration} + \text{Disease}$

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

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

23

## 24 Microscopy

25 To quantify glial sub-populations and neuropathological lesions in the pulvinar in a  
26 separate cohort of cases and  $\alpha$ -synuclein pathology in the amygdala of the cases  
27 used for the transcriptomic study, images were taken on a Zeiss AxioVision Z.1  
28 microscope using a DsFi1 camera (Nikon, Japan). As detailed previously (21, 30),  
29 Stereologer software was used to delineate a region of interest with a 2.5x objective,  
30 prior to placement of disector frames in a uniform, random arrangement. This

1 method prevented the introduction of bias by giving every area of the region of  
2 interest an equal probability of being sampled for analysis. Disector frame sizes were  
3 determined based on the size of the measured particles and their distribution across  
4 the region of interest. In all cases, amyloid- $\beta$  (4G8 anti-amyloid- $\beta$ , Covance, NJ,  
5 USA, 1:15000) was analyzed using 10x objective and  $\alpha$ -synuclein (5G4 anti- $\alpha$ -  
6 synuclein, Analytik Jena, Germany, 1:4500) and tau (AT8 anti-tau, Autogen, MA,  
7 USA, 1:4000); the microglial markers HLA-DP/DQ/DR (CR3/43, Dako, Denmark,  
8 1:1000), CD74 (LN-2, Santa Cruz, USA, 1:500) and Iba1 (Wako, Japan, 1:1000);  
9 and the astrocytic markers GFAP (Z0334, Dako, Denmark, 1:10000) and ALDH1L1  
10 (N103/39, Millipore, MA, USA, 1:7500) were measured using 20x objective.

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

20 These findings were correlated with densitometric analyses of neuropathological  
21 lesion burden to evaluate whether neuroglial marker expression was related to  
22 pathological protein deposition. A sub-set of cases used for histological analysis  
23 (8/14 control; 8/14 DLB) had been assessed as part of a previous stereological study  
24 of the pulvinar (21). Therefore, we additionally included stereological determination  
25 of total neuronal number within these analyses.

26

27

## 1 RESULTS

### 2 Demographic data

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

10 Demographic data for the histological analysis cohort is shown in Supplementary  
11 Table 2. There was no significant difference between groups in age at death  
12 ( $t=0.023$ ,  $df=26$ ,  $p=0.982$ ) or *post-mortem* interval ( $t=1.23$ ,  $df=26$ ,  $p=0.217$ ). There  
13 was no significant difference in the proportion of males relative to females between  
14 DLB and control ( $\chi^2=0.57$ ,  $df=1$ ,  $p=0.706$ ). Braak NFT was significantly higher in DLB  
15 cases compared to controls ( $t=3.88$ ,  $df=26$ ,  $p=0.001$ ).

16

### 17 Nomination of differential pulvinar gene expression between DLB and controls by 18 RNA sequencing

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

27

### 28 Validation of synaptic and immune proteins by western blot analysis

1 Analysis of protein expression using western blot analysis of general pre-synaptic  
2 markers demonstrated significantly lower expression of synaptophysin (U=30,  
3 p=0.015), NSF (U=25.5, p=0.006) and dynamin (U=37.5, p=0.047) in DLB compared  
4 to control (Figure 2). This was consistent with RNA-seq data, which demonstrated  
5 significantly lower expression of SYP (p=0.01), NSF (p=0.01) and DNMI1 (p=0.03).  
6 However, no significant differences were found in STX1A, SNAP25, SV2B or  
7 GAP43, despite significantly lower expression at the mRNA level.

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

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

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

28

## 29 Microscopy

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

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

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

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

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

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

6

### 7 Relationship between synaptic loss and neuropathological changes

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

14

### 15 Relationship between astrocytic increases and neuropathological, stereological and 16 synaptic changes

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

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

1 synaptophysin ( $r_s=-0.621$ ,  $p=0.041$ ), dynamin ( $r_s=-0.655$ ,  $p=0.029$ ), GABARAP ( $r_s=-$   
2  $0.673$ ,  $p=0.023$ ), and GAP43 ( $r_s=-0.627$ ,  $p=0.039$ ) in DLB cases.

3

#### 4 Clinico-pathological correlations

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

## 1 DISCUSSION

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

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

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

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

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

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

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

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

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

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

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5

6 AUTHOR CONTRIBUTIONS

7 Preparation of tissue homogenates and RNA isolation: DE and CMM

8 RNA sequencing and bioinformatics: JD, AK and MRC

9 Cutting of histological sections: AAK and DE

10 Neuropathological diagnosis of cases: JA

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

13 Interpretation of clinical notes: AJT, JPT and IGM

14 Preparation of manuscript: DE

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

17 Concept and funding of study: CMM, AAK and AJT

18

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6

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